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Investigating single platelet functionality using droplet microfluidics

by

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ABSTRACT

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INVESTIGATING SINGLE PLATELET FUNCTIONALITY USING DROPLET MICROFLUIDICS

Maaike Sybilla Anna Jongen

Platelet activation is an important step in arterial thrombosis, the acute complication of atherosclerosis. However, current diagnostic techniques for platelet function have been shown to be inadequate to predict thrombosis. Platelets have been shown to be heterogeneous in a number of features such as size, volume and density, and this variety may underpin overall system behaviour. Functional heterogeneity has been suggested in several studies, but current methods are not suitable to reliably study single platelet function. This study adapts a droplet microfluidics approach to investigate single platelet functionality.

Single platelet sensitivity is studied by adding the agonist (convulxin, specific ligand of the GPVI receptor for collagen) during encapsulation in droplets. After an incubation period the platelets are retrieved from the droplets into fixative, followed by flow cytometry analysis of markers for activation. The PAC-1 antibody is used to identify the active conformation of the αIIbβ3 receptor, important for aggregation and adhesion, and anti-CD62P (p-selectin) to identify degranulation of the platelets. Platelets are identified with CD42b, which is a platelet specific receptor.

This study has identified optimal droplet generation conditions, in terms of pinch geometry, flow velocity and flow ratio. These provide a means for the high throughput generation of monodisperse droplets and a significant number of droplets containing a single platelet. The platelet function can be analysed with PAC-1 and CD62P antibodies in a flow cytometer. The dose-response of convulxin is a sigmoidal curve. Platelets can be encapsulated within droplets without causing activation. Additionally, platelets can be stimulated with convulxin in droplets and this can be measured on a flow cytometer after breaking the emulsion. With this method an intrinsic variation in the platelet response to convulxin is observed, that is unrelated to the size of the platelet.

The research entailed the development of a method capable of measuring the intrinsic variation in platelet function. This presents the possibility to identify a novel prognostic biomarker. Furthermore, a better understanding of the functional heterogeneity of platelets could be used to identify new targets to aid the rational design of new therapeutics. Ideally, this would target only hyperactive platelets, while preserving normal haemostasis.

Table of Contents

Table of Tables

Table of Tables

Table of Figures

Table of Figures

Table of Figures

Table of Figures

Academic Thesis: Declaration Of Authorship

I, Maaike Sybilla Anna Jongen

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Investigating single platelet functionality using droplet microfluidics

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Chapter 1 Introduction

1.1 Platelet function

Thrombosis is a serious disease in which blood clots develop within blood vessels.[3] This restricts the blood flow to organs and tissues, which is potentially fatal. The three most lethal cardiovascular conditions, namely heart attack, stroke and venous thromboembolism, are caused by thrombosis.[3-5] The blood clots involved in thrombosis consist of aggregated platelets, stabilized with fibrin fibres.

Platelets are important to maintain homeostasis and prevent major blood loss by promoting haemostasis.[6] They are the smallest cellular component of blood, without nuclei and ranging in size between 1 and 5 µm.[7, 8] Platelets originate from megakaryocytes located in the bone marrow, whereby thousands of platelets are produced from a single megakaryocyte, whereby thousands of platelets are produced from a single megakaryocyte.[6, 9, 10] After up to 10 days, the platelets are cleared from the circulation.[11] A normal platelet count is between 150 and 450x10^9/L.[8]

Platelets promote haemostasis by forming the haemostatic plug, also called primary haemostasis, and by acting as a catalytic site for plasmatic coagulation, also known as secondary haemostasis.[12] Platelet function is a broad concept because platelets contribute to haemostasis in several ways. Platelet function can be defined in terms of adhesion, aggregation, morphology change, secretion and phosphatidylserine exposure. These terms will be discussed later.[12] Normally, in the absence of vascular injury, platelets circulate freely in a discoid shape and non-adherent state.[7] The undamaged endothelium produces inhibitors of platelet activation such as prostaglandin I_2 and nitric oxide and inhibitors of strong platelet agonists such as thrombin and ADP.[12] Apoptotic or procoagulant platelets are cleared from the circulation by the immune system.

At sites of vascular injury, the damaged endothelium can no longer suppress platelet activation and the subendothelial matrix is exposed to the blood.[12] The subendothelial matrix contains several adhesive components such as collagen and von Willebrand factor. Stable adhesion of platelets to these components leads to platelet activation.[12] Fibrillar collagen type I and III are the most important for the promotion of platelet adhesion and subsequent activation, while at conditions of high shear stress, von Willebrand factor plays a major role.[12, 13] Soluble von Willebrand factor binds to collagen fibres exposed in the subendothelial matrix, after which it can bind to platelets even in conditions of very high shear stress. The subendothelial matrix also contains tissue factor that starts the plasmatic coagulation cascade (secondary haemostasis), as discussed later.[14] Platelets bind to collagen mainly via the GPVI receptor and to von Willebrand factor via the GPIb-IX-V receptor complex.[13] The adhesive components in the subendothelial matrix cause an initial, unstable binding of platelets to the vessel wall. After tethering, rolling and activation, a complex signalling pathway leads to stable platelet adhesion (Figure 1-1).[12]

Platelet activation following adhesion to the subendothelial matrix leads to platelet granules fusing with the plasma membrane.[15] This fusion expels the contents of the granules into the extracellular space and increases the number of receptors on the plasma

membrane with the receptors stored on the granule membranes. Platelets contain three types of secretory organelles namely, alpha(α) and dense granules and lysosomes.[16] Of these secretory organelles, the α granules are the most abundant.

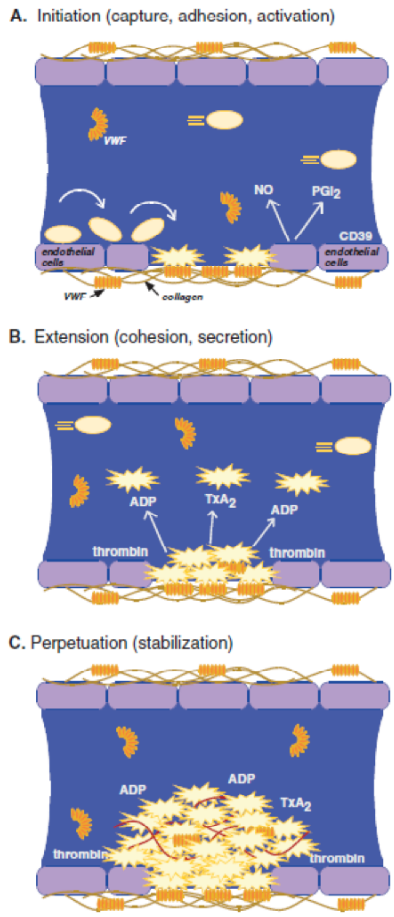


Figure 1-1 Steps in primary haemostatic plug formation. Michelson, Platelets 2006.[1]

Secretion products of α granules include components of the coagulation cascade (e.g. prothrombin, factor V, VII, XI, XIII and high molecular weight kininogens), inhibitors of fibrinolysis (fibrin is the end-product of secondary haemostasis) (e.g. plasminogen activator inhibitor-1 and α2-antiplasmin) and adhesive proteins (e.g. von Willebrand factor and fibrinogen).[17] The membrane of α granules also contains extra copies of important receptors like integrin $\alpha_{IIb}\beta_3$, so a significant increase in membrane receptor number can be observed after granule fusion. In addition to membrane proteins already present on the plasma membrane, the α granule contains membrane proteins only present on the plasma membrane after granule fusion such as p-selectin.[17]

Dense granules are more directly involved in the amplification of platelet activation.[16, 18] Dense granules secrete ADP, ATP, serotonin and calcium. Both ADP and serotonin are platelet activators, although ADP is more abundant and more potent than serotonin.[12] ADP binding to platelets liberates membrane-bound arachidonic acid, making it available for conversion into thromboxane A_2.[12] Thromboxane A_2 is a potent but short-lived platelet activator that is produced in activated platelets and released into the extracellular space, leading to amplification of platelet activation. So, secretion of granule contents will recruit and activate other platelets to the site of the growing thrombus and amplify the existing platelet response.

Platelet activation leads to a rise in cytosolic Ca^{2+} that cause platelets to change their shape from discoid to rounded and form pseudopods.[2, 12] This shape change increases the external surface area to facilitate contacts with other platelets and subendothelial matrix molecules. Elevated intracellular Ca^{2+} also leads to activation of the integrin $\alpha_{IIb}\beta_3$, the most abundant integrin on the platelet outer surface.[19] Platelet aggregation, the ultimate step of platelet activation, is achieved by crosslinking fibrinogen bound by integrin $\alpha_{IIb}\beta_3$.[20] In the resting state this receptor has a low affinity for fibrinogen that is increased by a conformational change following inside-out signalling.[12, 19] After activation, the integrin $\alpha_{IIb}\beta_3$ causes platelet aggregation and outside-in signalling. Outside-in signalling causes secondary secretion and amplifies the conversion of arachidonic acid into thromboxane A_2. The resulting platelet aggregate bound to the subendothelial matrix is also known as the primary haemostatic plug.

Finally, platelets contribute to the formation of a stable, fibrin-rich thrombus by interacting with the coagulation cascade, this process is called secondary haemostasis.[2, 14] Secondary haemostasis is a complex pathway eventually leading to the formation of fibrin fibres that

stabilize platelet aggregates and form stable clots. As discussed before, platelets secrete components of the coagulation cascade namely, prothrombin, factor V, VII, XI, XIII and high molecular weight kininogen and inhibitors of fibrinolysis such as plasminogen activator inhibitor-1 and α2-antiplasmin.[17] In addition, platelets form a negatively charged outer membrane by transporting phosphatidylserine from the inner leaflet of the plasma membrane to the outer leaflet.[21] The phospholipid composition of resting platelet almost exclusively consists of phosphatidylcholine and sphingomyelin on the outer leaflet, while the inner leaflet contains phosphatidylserine and phosphatidylethanolamine, not expressed on the outer leaflet. This phenomenon is known as lipid asymmetry.[21] This lipid asymmetry is lost, presenting phosphatidylserine and phosphatidylethanolamine to the outer leaflet, by Ca^{2+}-mediated activation of the transmembrane protein 16F (TMEM16F).[22] The phosphatidylserine exposure provides a catalytic surface for the cleavage of factor X by the complex of factor IXa and VIIIa.[2, 23] The coagulation cascade results in the formation of thrombin that cleaves fibrinogen into fibrin monomers that form fibrin fibres, subsequently cross-linked by XIIIa.[14] In addition, the formed thrombin is a potent activator of platelet aggregation.[13] Therefore, it can be concluded that platelet activation and blood coagulation enhance each other and haemostasis is a combination of both processes.[2]

1.2 Thrombosis

Thrombosis is the pathological process in which a thrombus forms inside a vessel rather than on the edge of a damaged vessel.[3] This restricts the blood flow to organs and tissues, which is potentially fatal. The three most lethal cardiovascular conditions, namely heart attack, stroke and venous thromboembolism, are caused by thrombosis.[3-5] The blood clots involved in thrombosis consist of aggregated platelets, stabilized with fibrin fibres. Two major types of thrombosis exist, named after their position in the body and with different pathology, namely, arterial thrombosis and venous thromboembolism. The thrombi formed by these two processes look different. Traditionally, the clots involved in venous thromboembolism are described as "red" clots, these contain a lot of fibrin fibres which trap red cells in the growing thrombus and arterial thrombosis clots are described as "white" clots, that are rich in platelets but don't contain the large numbers of red cells seen in venous clots.[24] While recently, these two forms of thrombosis have been shown more linked than previously expected, from a platelet (function) context, the arterial thrombosis is more relevant than venous thromboembolism.[24]

Arterial thrombotic diseases are responsible of more than 25% of all deaths worldwide.[4] The main cause of arterial thrombosis is atherosclerosis. When an atherosclerotic plaque ruptures, the subendothelial matrix is exposed to the blood, which triggers platelet adhesion and activation. The main risk factors involved in arterial thrombosis are those associated with more inflamed and rupture-prone atherosclerotic plaques such as obesity, diabetes and smoking. However, it is also known that these risk factors also create a more hyper-active platelet profile which is also associated with non-response to anti-platelet treatments such as aspirin and clopidogrel.[25, 26] More recently it was discovered that platelets accelerate the formation of atherosclerotic plaques.[3, 4] Additionally, the asymptomatic micro-thrombi observed in the arterial circulation of individuals with these risk factors cause an acute inflammatory process within the (chronically inflamed) atherosclerotic plaques. This dramatically increases the risk of plaque rupture and associated vessel occlusion. While the identification of these risk factors has improved the selection of individuals needing treatment to slow down the progress of atherosclerosis,

the identification of individuals truly at risk for arterial thrombosis remains a challenge. Furthermore, all current treatments and prevention of arterial thrombosis (not the prevention of the underlying atherosclerosis) are associated with increased risks of bleeding events.[13, 27, 28] And most of the current treatments include a relatively large number of non-responders. Therefore, it is important to improve the identification of people at risk and which therapy is most effective for them.

Platelet function is a collection of several connected processes that all contribute to haemostasis. As such, platelets have an important role in preventing major blood loss. Furthermore, many different platelet disorders have been described with mild to severe bleeding diathesis.[29] However, platelets are also involved in the pathology of thrombosis and the treatment and prevention of thrombosis often involves platelet inhibitors.[13] Although effective treatments for thrombosis have been developed, most induce mild to severe bleeding.[13, 27, 28] Effective treatments for thrombosis without bleeding side effects have not been successfully developed. On the other hand, treatment for bleeding disorders often leads to increased risk of thrombosis. Because of the consequences and risks of (anti-)platelet therapy, careful diagnosis of platelet function is necessary. However, while diagnostic methods can detect bleeding tendencies reasonably well, no clinical method is currently available that tests the thrombotic tendencies.[4, 30-32] Further research is required to develop further diagnostic tests and new therapies.

The recent studies investigating platelet response heterogeneity have identified new targets for treatments. For example, as suggested by Munnix et al the specific targeting of only the procoagulant platelets could limit excessive thrombus formation without interfering with platelet aggregation.[33] Furthermore, the elucidation of intrinsic functional heterogeneity could potentially improve the identification of individuals at risk for arterial thrombosis, and represents the focus of this study.

1.3 Platelet heterogeneity

The heterogeneity of platelets has long since been a topic of interest. At first, heterogeneity in size, volume, organelle distribution and receptor density were studied.[34-37] Heterogeneity was studied later with several aspects of platelet heterogeneity linked to function or functional heterogeneity being investigated.[38] Some of these heterogeneous aspects may originate from megakaryocyte heterogeneity.[39, 40] Furthermore, a large source of heterogeneity of density and function seems related to the platelet age, where younger platelets seem to be bigger and have more functional capacity.[41-44]

In 2000, a new type of platelet was described by Alberio et al, named COAT platelets, because they are found in response to COllagen And Thrombin stimulation.[45] These platelets have phosphatidylserine (PS) exposure and bind coagulation factors such as factor V. Furthermore, they were shown to have deactivated $\alpha_{IIb}\beta_3$ integrin on their surface.[46, 47] Later these were called COATED platelets instead and have been studied by many groups since.[33, 48-54]

Before the description of COAT platelets, procoagulant activity in platelets was described in a population of ballooning platelets.[50] These platelets show a large cavity (the balloon) surrounded by the plasma membrane and a small dense area, sometimes described as a cap, thought to be the platelet body.[55, 56] The cap was shown have phosphatidylserine (PS) exposure that bound coagulation factors, thereby providing a pro-coagulant surface. The

ballooning was shown to be dependent on sustained high cytosolic Ca^{2+} levels that activated salt incorporation within the platelet followed by large quantities of water.

However, the in vivo relevance of these types of heterogeneity is still unknown. Using intravital microscopy it was discovered that a thrombus contains areas of strongly active platelets towards the vessel wall, the core, and less active, loosely attached platelets around this core, the shell.[57, 58] For instance, platelets positive for P-selectin are located in the core but not the shell. P-selectin, however, is not related to the coated platelet heterogeneity and sometimes used as an overall activation label separate from the pro-coagulant activity.

Overall the most extensively studied heterogeneous population is the COAT/coated platelet population.[59] However, a whole variety of functional heterogeneity have been described such as, ballooned platelets, BAPS (Ballooned and Procoagulant-Spread platelets), SCIP (Sustained Calcium-Induced Platelet morphology), MPTP (Mitochondrial Permeability Transition Pore phenotype), GSAO (4-[N-(S-glutathionylacetyl) amino] phenylarsonic acid binding platelets) and FIB-CAP (Fibrinogen Capped Platelets). Recently however, it was proposed by Agbani et al[60] all these heterogeneous platelet populations are actually the same population with versatile platelets of which the most important characteristic is that they are procoagulant and proposed a general name of procoagulant platelets. While most characteristics are the same for suspension as well as adhesion models, procoagulant spreading was only observed in adhesion models.[60]

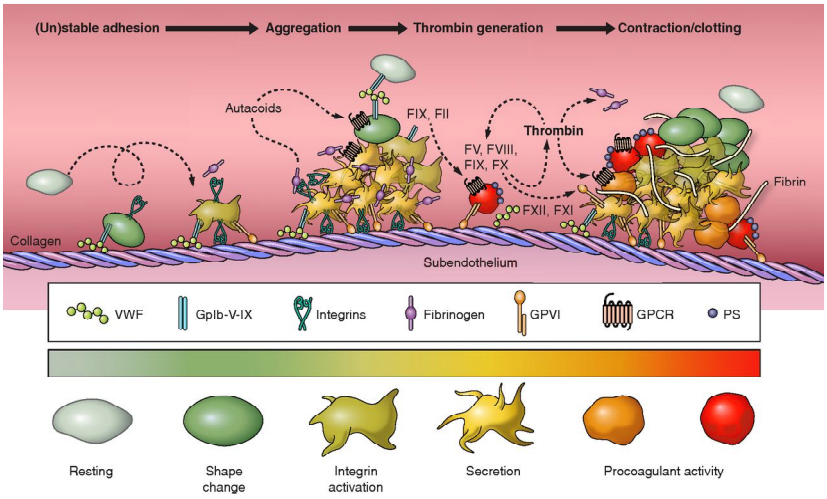


Figure 1-2 Spatiotemporal heterogeneity in platelet response. The response of a platelet to an activating stimulus (collagen) is dependent on the time and the place of activation within a growing thrombus. Strongly adherent platelets get activated by contact with the collagen and are thus located in the centre of the thrombus (yellow). Later recruited platelets have a lower binding affinity (green) or a procoagulant profile (red). Versteeg 2013[2]

1.4 Models of platelet function

A wide variety of methods and targets are available to study platelets and platelet function however it is important to make a distinction of suspension or adhesion models and consider the advantages and limitations of each method. Here a few of the most relevant and commonly used methods are reviewed.

1.4.1 Aggregometry

The preferred method for (diagnostic) platelet function testing is light transmission aggregometry (LTA), mostly accepted as the gold standard method for platelet function testing.[8, 61] The test was developed in 1962 by Born and O'Brien.[31, 62-64] LTA measures the decrease in optical density of a clear solution containing platelets, usually platelet rich plasma or washed platelets, during aggregation in response to an agonist.[62] Platelet rich plasma without agonist serves as a negative control (0%; low light transmission) and platelet poor plasma from the same donor and also without agonist as a positive control (100%; high light transmission).[61] During the test, the platelet rich plasma is stirred (with low shear) constantly and kept at a constant temperature of 37 °C.[61, 62, 65] As such it is a suspension test of platelet function. Following addition of an agonist, a brief small increase in optical density takes place because of the shape change of the platelets in the sample[62], although some agonists cause only a small shape change curve or none at all. After the shape change, the optical density decreases because of the first wave of aggregation. The second wave is faster and more extensive and is dependent on thromboxane A_2 production and granule release. Stimulation with some weak agonists in low concentrations (e.g. <2.5 µM ADP) does not result in a second wave but in disaggregation.[62] Stimulation with strong agonists (e.g. collagen) causes the first and second waves to fuse together so that they cannot be distinguished. Different disorders lead to different aggregation curves after stimulation with a panel of agonists.[66]

Platelet aggregation can also be analysed in whole blood using impedance measurements. When platelets adhere to the two electrodes, the impedance between them changes.[67, 68] Because aggregation can be measured in whole blood, no centrifugation of the blood is required so no artificial activation of platelets due to centrifugation takes place and bigger platelets are included.[67, 69] Furthermore, red blood cells have a modulating effect on platelet aggregation which is taken into account.[69] However, aggregation is influenced by low haematocrit and elevated white count.[66]

Modern aggregometry can be combined with luminescence analysis of platelet ATP secretion, which is useful because many (mild) platelet function defects are associated with decreased secretion.[32, 70] This assay can be performed in both platelet rich plasma and whole blood in combination with optical aggregometry and impedance aggregometry respectively.[69-72] The lumiaggregometry method is based on the ATP release from the platelet dense granules, which acts as a co-factor in the enzymatic conversion of luciferin by firefly luciferase.[71] In this reaction, the luciferin is oxidatively decarboxylated, which results in light emission. The light emitted is proportional to the amount of ATP released from the platelets. This method causes the simultaneous evaluation of aggregation and ATP secretion without requiring radioactive probes. However, a large within- and between-subject variability has been shown.[32, 70] Moreover, the presence of the most commonly used reagent (Chronolume) potentially leads to a normal LTA result in patients with common platelet function disorders already diagnosed by standard LTA.[32, 73, 74]

Other diagnostic tests such as the platelet function analyser are available that are capable of testing under different conditions like shear stress or combinations in agonists.[8] However, these tests have been developed to diagnose platelet disorders causing bleeding diathesis and are neither designed nor suitable for diagnosing thrombotic tendencies.[4, 30, 31] The development of diagnostic tests capable of predicting thrombosis requires further (fundamental) research into the roles of platelets in normal and pathological thrombus formation. However, diagnostic tool development also needs a focus on being useful for

clinical application (costs, reliability, sample volume, ease of use and speed).[8] These considerations are mostly not relevant when studying platelet function of healthy volunteers for fundamental research and are beyond the scope of this review.

1.4.2 Flow cytometry

Flow cytometry is an incredibly versatile tool for measuring platelet function because it can measure several outcome measures, and is suitable for both whole blood and platelet rich plasma.[75] A flow cytometer can analyse specific characteristics on individual cells with a high throughput (normally max 20,000 cells/sec). To analyse platelet activation, a diluted platelet suspension is activated with an agonist and labelled with a platelet specific label and an activation label.[75, 76] After an incubation time, usually between 5 and 15 minutes, the platelets pass through a flow cell with a laser beam and detectors to measure light scattering and the fluorescence emission.[75] The forward light scatter is proportional to the size of the cells, while the sideward light scatter is proportional to the granularity. In a whole blood sample, the light scatter pattern can be used to distinguish platelets from the other blood cells but the use of a platelet specific antibody is recommended to exclude platelet-sized cellular fragments and other similarly-sized particles.[75-77] Depending on the choice of antibody, several aspects of platelet activation can be studied such as integrin $\alpha_{IIb}\beta_3$ activation, granule secretion and phosphatidylserine exposure that results from membrane inversion. Integrin activation, as a marker for aggregation, can be studied with antibodies that are specific for the fibrinogen binding site because this is only accessible on activated platelets.[78] The preferred antibody is PAC-1, although fluorescently labelled fibrinogen can also be used but the pool of unlabelled endogenous fibrinogen has to be considered.[19, 75] Many markers for granule secretion are available but p-selectin, an α granule membrane protein, and CD63, a dense granule (and lysosome) membrane protein are the most commonly used.[17, 18] Both are only present on the plasma membrane after granule fusion. However, CD63 is less sensitive due to a lower copy number and a greater level of platelet activation is required before it can be measured. Alternatively, phosphatidylserine exposure a feature of membrane inversion during platelet activation can be measured using annexin V that binds to phosphatidylserine.[75]

Advantages of flow cytometry include small sample volumes, wide choice of outcome measurements and importantly that the platelets are analysed individually.[76] Moreover, whole blood flow cytometry limits platelet activation due to centrifugation. Fixing platelets after activation allows delayed flow cytometry analysis for convenience. Even though platelets are analysed individually, during activation recruitment and activation can still take place as a result of platelet-to-platelet interactions, such that platelet-platelet extrinsic effects cannot be excluded.

1.4.3 Intravital microscopy

Innovations in intravital microscopy, studying thrombus formation after an induced vascular injury in a living animal (usually a mouse), including the development of platelet labels and two-photon microscopy have revealed heterogeneity in response.[58, 79] Using these techniques it was discovered that a thrombus contains areas of strongly active platelets towards the vessel wall, the core, and less active, loosely attached platelets around this core, the shell.[57, 58]

However, while many aspects of platelet function are well preserved in evolution some differences between animals and humans are present. For example, it has been described that human platelets spread on a fibrinogen surface as well as a collagen surface while mouse platelets only spread on collagen and not on fibrinogen.[80] Verification of findings in mouse models in humans remains necessary. One way to study thrombus formation with human platelets is in the use of parallel-plate flow chambers.

1.4.4 Parallel-plate flow chambers

To study platelet function (for research purposes) in a more natural environment than the standard diagnostic tests described above, the parallel-plate flow chamber was developed.[81] In this method, minimally handled blood (i.e. no centrifuge, no storage, only anticoagulant etc.) is flowed over a surface coated with a platelet agonist (e.g. collagen) and a thrombus is allowed to develop.[82] This method is used for both animal and human platelets studies, although it can be seen as a way to decrease the need of animal models. The size of the thrombus is limited by the size of the channel inside the chamber and the surface area that is coated.[83] By adding fluorescent labels, thrombus formation can be followed in real-time with a fluorescent microscope and even in 3D with a confocal microscope. The response of different platelets can be monitored at different sites within a growing thrombus.[33, 53] This method can control shear rate, so the effects of shear stress on the size and consistency of a thrombus can by investigated.[84, 85] The adhesive qualities of different surfaces can be investigated and it is even possible to coat the surface with several agonists at different spots to screen for more than one platelet defect simultaneously.[86] Additionally, the effects of coagulation on a growing thrombus can be studied by counteracting the anti-coagulant and adding an appropriate agonist, such as tissue factor.[87-89] This method has improved our understanding of the role of platelets in thrombus formation greatly although there is a strong call for standardization.[90, 91] This method is also essential in the study of spatiotemporal heterogeneity of platelet response.[33, 53]

1.4.5 Novel techniques using miniaturisation

In 2009 a group of researchers developed a method similar to parallel plate flow chambers that used a microfluidic device with a channel containing a different degree of stenosis.[92, 93] This technique is especially useful because of the adjustable but controlled degree of stenosis. The miniaturization offers an in depth view of a growing thrombus at a site with significant stenosis.[94] Other adaptations use several parallel flow chambers to study several conditions/stimuli simultaneously or different types of stenosis.[95-98] Miniaturisation is used in several other methods.[99] The rapidly expanding field of microfluidics provides a new concept of studying platelet function. These methods promise an unprecedented level of control, over shear, exposure to stimuli, and separation of specific platelets while minimising the costs of agonist and sample volumes because of the miniaturisation.[99-102]

Deterministic lateral displacement is a label-free method to separate active from non-active platelets based on the shape change.[103] When platelets are activated, they create pseudopods and then spreading in between these. This increase in effective hydrodynamic size means they are displaced in this microfluidic device and thus can be separated. While this is an effective and cost-efficient way to study platelets, it is mostly useful in situations where a fast estimation of platelet function is needed but without access to a laboratory.

However, DLD involves repeated collisions and high shear stress which can cause artificial activation. Furthermore, it is less valuable than other tests because the shape change is not the most clinically relevant parameter. However, shape change is hard to measure using the standard tests and is therefore not as fully been investigated as other aspects of platelet activation. Another method focusing on the shape change of active platelets is based upon the microscopic investigation of platelets adhering and spreading on a coating done with microcontact printing.[104] However, this approach is laborious and not precise.

While all these different approaches have led to increased understanding of the mechanisms underlying platelet function, single platelet functionality and the heterogeneity within the population has not been investigated with these new techniques. That is to say that while some of these studies look at the response on a single cell level with microscopy, the activation step occurs in (close) contact with other platelets, so paracrine signalling and amplification is present. This means that intrinsic heterogeneity cannot be studied because spatiotemporal heterogeneity cannot be excluded. Moreover, these studies have not solved the problems in predicting thrombotic events or their possible complications.[4, 105] Currently, there is no method that can study the behaviour of single platelets, without influence of other platelets in the vicinity. Nevertheless, research on other types of cells has shown that not all cells from the same cell type are the same.[106] For example the fluctuations of heterogeneous populations of progenitor stem cells[107] and the diverse responses of lymphocytes.[108, 109] Additionally, studies of pro-coagulant platelets have strongly suggested a role for both spatiotemporal and intrinsic platelet response heterogeneity.[33] However, a definitive conclusion of intrinsic heterogeneity cannot be made without studying the response of platelets completely separated from the influence of other platelets.

1.5 Droplet microfluidics

The challenge of completely isolating platelets during the activation process can be solved by individually encapsulating them within droplets. An emulsion of droplets is formed by mixing an aqueous phase with an oil phase.[110] In recent years, microfluidics has become the preferred method to form droplets as uniform forces are exerted when oil flows merge with aqueous flows for the generation of monodisperse (uniformly-sized) droplets typically with a <5% coefficient of variation.[111] Microfluidic droplets are typically 5-50 microns in diameter producing picolitre volume compartments. Several designs have been described for the formation of the droplets with the most important part of the device being the junction where the hydrophobic and hydrophilic phases meet. Two major types of junctions exist, the T-junction, where the hydrophobic phase is interrupted by a perpendicular channel for the hydrophilic phase, and a flow focusing junction, where the hydrophilic stream is engulfed from both sides by the hydrophobic phase.[112, 113] To prevent droplet coalescence, the interface is stabilised with a surfactant added to the oil phase.[113] Most of these devices are made from PDMS (polydimethylsiloxane) which is suitable for the rapid and cost effective replication of devices in biology laboratories, is transparent for imaging and which allows the exchange of oxygen and carbon dioxide gases from as required for live cell assays.[114] Furthermore, fluorinated oils can be used that facilitate gas exchange to produce a life support environment suitable for lengthy containment of cells without affecting their behaviour. For example, droplets have been used for small scale cell culture over relatively long periods of time (>1 month).[113] Relevant to my research, Song et al. demonstrated that blood coagulation can still take place within a droplet by applying the

activated partial thromboplastin time (APTT) test.[115] When using droplet microfluidics to study single platelet function, platelets need to be activated (by soluble agonists) as they are rapidly encapsulated. Care should be taken that encapsulation is rapid enough to avoid degranulation (activation and recruitment of other platelets) and aggregation. Studying single platelet sensitivity to activating stimuli can therefore be achieved using droplet microfluidics as the rapid compartmentalisation process prevents cross-talk by paracrine signalling.

Another merit of droplet microfluidics is the high throughput potential, with typical droplet generation rates of 0.1 and 10 kHz.[110] However, to obtain droplets that contain only one cell Poisson effects come into play, necessitating sample dilution to ensure single cell encapsulation while the majority of other droplets contain no cells (e.g. 85-95%).[116, 117] Spatial ordering by an inertial process called microfluidic entrainment can be used prior to encapsulation and this will markedly increase the number of droplets containing a cell, without appreciably increasing the number of droplets with more than one cell.[116] This approach can therefore increase the throughput of the platelet encapsulation process, although working at the scale of a platelet is particularly challenging for this branch of microfluidics.

After encapsulation the platelets within the droplets can be studied for activation in several ways; one option is to stain for an activation marker and count the active platelets using a fluorescent microscope. However, microscopy measurements of single picolitre droplets is a time-consuming process, involving high magnification imaging and often lengthy exposure times, overall limiting the throughput of the overall analytical process despite the high frequency generation rates. Alternatively, single platelets can be analysed in high throughput using a flow cytometer (max ≥20,000 cells/sec). However, the carrier fluid for the droplets is fluorinated oil that is incompatible with a flow cytometer which requires aqueous carrier fluids. To solve this problem methods have been reported in the literature. One solution involves introducing the droplets into a second droplet generating microfluidic system to form a water-in-oil-in-water double emulsion.[118] These double emulsions were also shown to be monodisperse, biocompatible and stable. Because the carrier solution is aqueous these double emulsion droplets are suitable for analysis by flow cytometry. Another solution involves gelation where polymer molecules such as agarose or alginate are introduced during droplet formation.[119] Finally, the emulsion can be destabilized to retrieve the platelet from the droplets after a certain incubation time.[120] If the emulsion is broken in the presence of fixative, the activation states of the platelets are maintained. All of these approaches access the high throughput of flow cytometry analysis that matches the high throughput of the droplet generation process, overall producing a straightforward work flow that can be used to analyse many thousands of platelets to produce statistically relevant data sets even for the detection of rare events.

Overall droplet microfluidics as discussed here provides a simple means for the rapid screening of large numbers of platelets to effectively survey the variety within the population for the identification of possibly rare and potentially highly influential platelets. Therefore, this method is especially useful for the elucidation of the platelet heterogeneous response question. My PhD project aims to address the hypothesis that platelet sensitivity to activating stimuli varies between single platelets within individuals and this affects thrombus formation both in health and in disease.

Chapter 2 Assay development

2.1 Introduction

Platelet function can be studied in several ways, although identifying individuals at risk of developing thrombosis or thrombotic complications of certain types of surgery is still complicated.[105, 121, 122] In recent years, emerging technical developments have resulted in an increased interest in the study of platelets by means of microfluidic techniques.[92, 94-96, 99, 103, 104, 123-126] Most of these methods have focussed on improving in vitro thrombus formation to be as physiologically accurate while allowing specific manipulations to study the effect of for example atherosclerosis on thrombus formation.[83, 94, 99] Others have been designed to answer specific questions of platelet function in health and disease, such as spreading or agonist gradients.[100, 103, 104, 127] While all these different approaches have led to increased understanding of the mechanisms underlying platelet function, single platelet functionality has not been researched with these new techniques. Moreover, these studies have not solved the problems in predicting thrombotic events or their possible complications.[4, 105, 128] Encapsulating individual platelets in droplets eliminates the platelet-platelet interaction and amplification. Furthermore, droplet formation is monodisperse and high throughput despite the constraints of Poisson statistics.[110, 111, 116, 117] Matching the high throughput stimulation of single platelets with the high throughput analysis of flow cytometry allows for the study of variety and rare events in responses.

Platelets are increasingly studied with flow cytometry because it is a very versatile method that can be adjusted for multiple purposes.[75, 129] Flow cytometry is broadly applicable because of the wide range of markers that can be targeted with specific antibodies and even the simultaneous detection of different markers to study different aspects of the same platelet.[75, 78, 129, 130] Despite the fact that flow cytometry is not suitable to measure platelet aggregation, it can measure the integrin activation needed for aggregation (i.e. the $\alpha_{IIb}\beta_3$ receptor).[131] In resting platelets, this receptor is present but is not able to bind fibrinogen, but upon activation of the platelet, the receptor undergoes a conformational change after which it is able to bind fibrinogen.[19, 76] Platelet activation via the activation of the $\alpha_{IIb}\beta_3$ receptor can be measured by PAC-1, a monoclonal antibody that binds the fibrinogen binding site. [132-134] Degranulation is also a result of platelet activation and leads to the presentation of granule membrane proteins on the plasma membrane.[17] P-selectin (CD62P) is one of these membrane proteins (of α-granules) and is currently the most studied because it is highly expressed but dense granule secretion (and lysosomes) is frequently tested by CD63 expression.[131, 133, 135, 136] For flow cytometric applications, a platelet specific antibody is often added to better distinguish between platelets and other cell debris, such as CD61 or CD42b.[78, 133, 137] While aggregation is a result of stimulation by most agonists, degranulation is only triggered by some agonists.[133] Collagen is one of the agonists that leads to both aggregation and degranulation.[138, 139] In flow cytometric assays convulxin, a specific activator of the main collagen receptor (the GPVI receptor) is often used.[140]

This study uses droplet microfluidics and flow cytometry to investigate single platelet functionality. Some research on COAT platelets (collagen and thrombin activated platelets, that show some distinct populations with regards to phosphatidylserine exposure, among other processes) suggests there is a functional difference between single platelets.[33, 48, 49,

[52] However, these studies involved stimulating the platelet population under conditions where they can interact (i.e. in presence of paracrine signalling). Therefore, single platelet functionality cannot be investigated without ruling out the effects of paracrine signalling that amplifies platelet responses, that mask the unique functional capacity of individual platelets.[141] Encapsulation of platelets in a droplet compartment and subsequent analysis using flow cytometry allows for the investigation of single platelet function without platelet-platelet interaction (paracrine signalling). However, the combination of both methods requires adaptation and optimization.[142] Therefore, this study aims to:

- Design a device able to produce monodisperse droplets in a high throughput manner.
- Optimize droplet formation for the encapsulation of single platelets.
- Optimize the antibody panels for the simultaneous measurements of multiple aspects of platelet function.
- Integrate and optimize a droplet microfluidics approach with flow cytometry to study single platelet function.

2.2 Methods

2.2.1 Materials

Fluorescent staining was achieved with fluorescein isothiocyanate (FITC) conjugated PAC-1 (PAC-1 clone), allophycocyanin (APC) and FITC conjugated CD62P (P-selectin) (AK-4 clone), FITC conjugated anti-CD63 (H5C6 clone) PE and PerCP-Cy5.5 conjugated CD61 (VI-PL2) and R-phycoerythrin (PE) conjugated CD42b (HIP1 clone) obtained from Becton Dickinson Pharmingen (San Jose, California, United States). Platelets were activated with convulxin, a snake venom toxin specifically activating the GPVI receptor (main collagen receptor), obtained from Enzo Life Sciences (Exeter, United Kingdom). Blood samples were diluted in HEPES buffer containing 136 mM NaCl, 2.7 mM KCl, 10 mM HEPES and 2 mM $MgCl_2$ (pH 7.45), stored at 4-7 °C, with glucose 0.1% w/v and BSA 1% w/v added freshly before use.

2.2.2 Design

The designs in this study use flow focusing junctions to form the droplets. Several different flow focusing junctions are described in the literature.[112, 143] Initially, 4 different junctions were tested (Figure 2-1). The width and height of the channel are influenced by the size of the cells, in this case platelets with a size of 1-4 µm, and the Poisson statistics that determine the optimal size of the droplets for single platelet analysis (Figure 2-4).[116, 120, 144] In this case droplets with a diameter of ~18 µm would be optimal for single cell encapsulation. Therefore, pinch sections were designed with a diameter of 22 µm. The height of the devices, dictated by SU-8 spin-coating results, was 19 µm for Figure 2-1A-D and 20 µm for E and F.

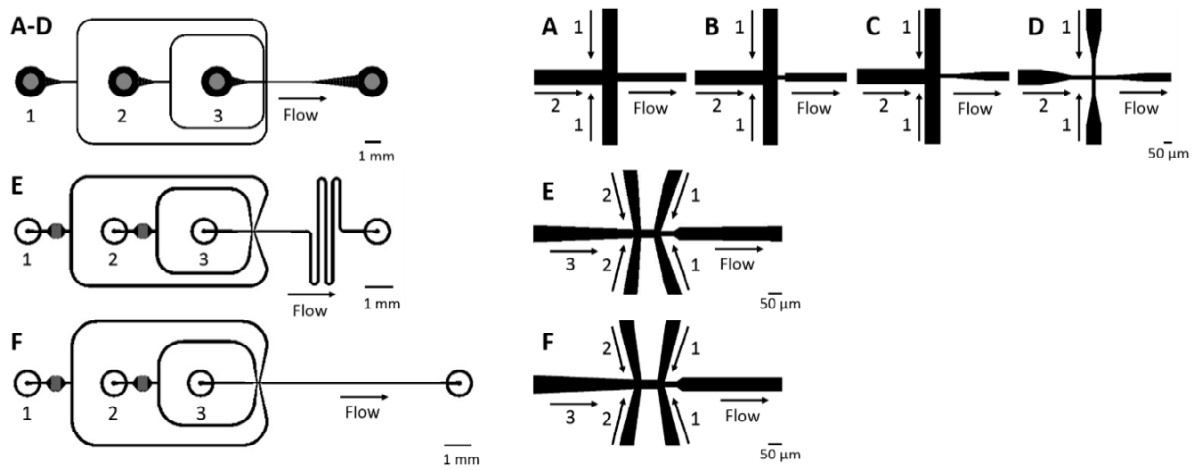


Figure 2-1 The devices used for the formation of droplets with their respective pinch geometry.

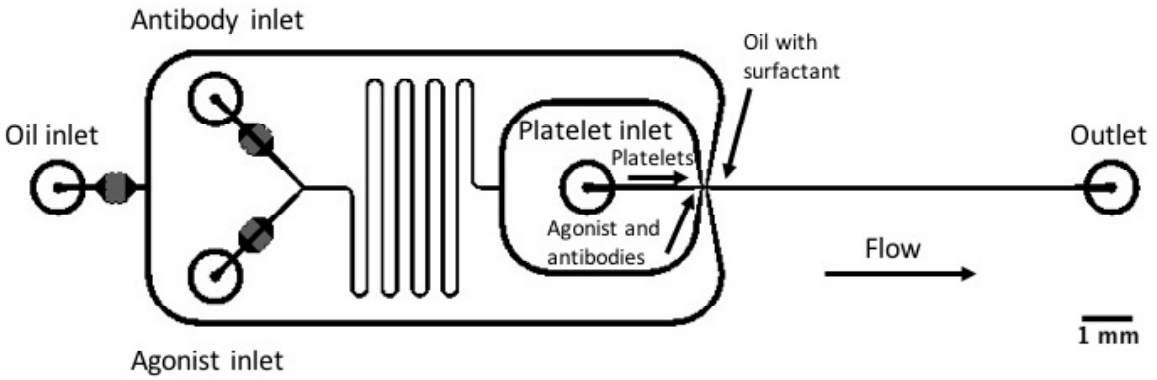


Figure 2-2 Final design of the device used for single platelet studies.

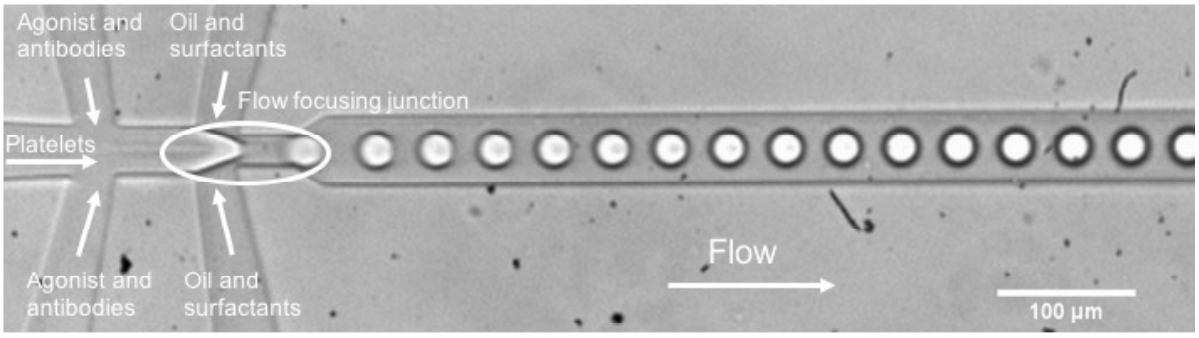


Figure 2-3 Close-up of the flow focusing junction of the device depicted in Figure 2-2.

2.2.3 Device fabrication

The devices were fabricated using a standard photolithography protocol.[145, 146] Briefly, the design was made using DraftSight software (Dassault Systèmes Solidworks, Waltham, Massachusetts, United States). From this design an UV photolithography mask, prepared by e-beam lithography of chrome on soda lime glass, was manufactured by JD PhotoTools. An SU-8 photoresist-coated silicon wafer was exposed to UV radiation while shielded by the mask. After solvent-based development of the photoresist a master with the specific 2-D design extending from the surface of the silicon wafer was prepared.

The master was replicated into polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, Michigan, United States) by pouring approximately 5 grams of PDMS onto the master, contained within a Delrin frame and left to cure for approximately 45 minutes at

80°C. This PDMS device was punched with a 1-mm-diameter biopsy punch (Miltex biopsy punch, Integra, Plainsboro, New Jersey, United States) to create the inlets and outlets. Devices were cleaned with 3M tape to remove particles without leaving residues. Subsequently, the device was bonded to a glass coverslip coated with a thin layer (<1 mm) of PDMS by oxygen plasma activation (30 seconds) of both PDMS surfaces using a Diener Asher device (Femto, Diener Eletronic, Germany), to cover the microchannels. These hydrophilic microchannels were coated with Trichloro(1H,1H,2H,2H-perfluorooctyl)silane (Sigma Aldrich, St. Louis, Missouri, United States) 1% v/v in HFE-7500 (3M™ Novec™ 7500 Engineered Fluid), to covalently silanise the channels, rendering them hydrophobic and suitable for the oil carrier phase. After coating, the channels were dried with N_2 and subsequently flushed with HFE-7500.

2.2.4 Droplet generation

For droplet generation, fluorinated, bio-compatible oil and surfactant were used, especially designed for live cell assays (0.75% EA in HFE-7500, Ran biotechnologies Beverly, Massachusetts, United States). Fine bore polythene tubing with an outer diameter of 1.09 mm and an inner diameter of 0.38 mm (Smiths Medical International, Hythe, United Kingdom) was inserted into the inlets for straightforward plug and play operation. No additional interconnection procedures were used, to streamline the assembly process. Pulse free syringe pumps (Fusion 200, Chemyx, Stafford, Texas, United States) were used to infuse the solutions into the devices. A total flow rate of 900 µL/hr and, unless otherwise stated, a flow ratio of water to oil of 1:4 was used.

Encapsulation was tested using Ø 2.1 µm fluorescent polystyrene latex microspheres (Fluoresbrite®, Polysciences, Warrington, Pennsylvania, United States), in the device shown in Figure 2-1D. Live droplet generation was monitored and recorded using a high speed camera (Vision Research, Wayne, New Jersey, United States) and bespoke software (Phantom Camera Control Software, Vision Research).

2.2.5 Participants

Blood was obtained by venepuncture from healthy volunteers after obtaining written informed consent. Ethics approval was obtained from the NRES Committee South Central – Hampshire B (REC reference 14/SC/0211) and from ERGO (protocol number 5538). All participants were free from anti-platelet medication, such as aspirin for 2 weeks and 24 hours free from other non-steroidal anti-inflammatory drugs. Volunteers were evenly distributed among genders (5 males and 3 females) and ages (3 between 20 and 30, 2 each for 30-40 and 40-50 and 1 between 50 and 60). The blood was collected using a 21 G needle, into Vacuette tubes containing 1:10 v/v 3.2% trisodium citrate (0.109 M) and gently inverted three times. The first 4 mL was collected into a K_2EDTA containing Vacutainer® tube (BD, Franklin Lakes, New Jersey, United States) and only used for platelet counts.

2.2.6 Blood preparation

Directly after blood collection, citrate tubes were centrifuged at 240 g for 15 min without a brake to prepare platelet rich plasma (PRP). The PRP was set aside for approximately half an hour before use. In the meantime, the platelet count was determined (see section 2.2.7). Subsequently, platelet counts in PRP were adjusted to $100x10^6$/mL and the platelet count measured using the same protocol. Finally, the platelet count adjusted PRP was diluted 1:4 in buffer (final concentration $25x10^6$/mL) and loaded in the syringe or used for controls.

Table 2-1 The antibody panels used by this study and their respective concentrations.

		Conjugated with		
		FITC	PE	APC
Antibody panel	A	PAC-1 1.25 ng/µL	CD42b 1.25 ng/µL	P-selectin 0.63 ng/µL
	B	PAC-1 1.25 ng/µL	CD42b 1.25 ng/µL	Annexin V 0.08 ng/µL
	C	P-selectin 2 ng/µL	CD61 0.25 ng/µL	Annexin V 0.08 ng/µL
	D	CD63 2 ng/µL	CD61 0.25 ng/µL	P-selectin 0.63 ng/µL

2.2.7 Platelet counting

The platelet count in the PRP and EDTA anti-coagulated whole blood was determined using a protocol by Masters and Harrison, 2014.[147] Briefly, 20 µL of a 20-fold diluted PRP (in HEPES buffer) was stained for 15 min with 1 µL anti-CD61. After the incubation time, this stained sample was further diluted 50-fold. An Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, United States) was used to analyse 100 µL of this diluted sample and platelet counts were calculated from the number of positive events.

2.2.8 Statistical analysis

Droplet videos were analysed using ImageJ software (open scource[29]). The area of the droplets was calculated automatically using this software. The Shapiro-Wilk test was used to verify normality and in instances where this was significant non-parametric statistics were used. The droplet sizes were reported with the median and the quartile coefficient of dispersion (QCD). The QCD is calculated with the equation: $\frac{Q3-Q1}{Q3+Q1}$ where Q3 is the third quartile (75%) and Q1 is the first quartile (25%). Non-parametric univariate analysis of single platelets consisted of median with interquartile range. Significance testing of single platelet experiments was performed with the relative risk / risk ratio procedure plus confidence intervals. For these single platelet experiments this test was named relative activation.

2.3 Results

2.3.1 Optimal droplet size for single object encapsulation can be predicted by Poisson statistics

Single cell encapsulation is highly dependent on the Poisson distribution[116, 117], which describes the probability of the number of cells within a droplet as a function of the number of cells in a droplet (n) and the average number of cells in a droplet (λ):

$$P(n,\lambda) = \frac{\lambda^n e^{-\lambda}}{n!}$$

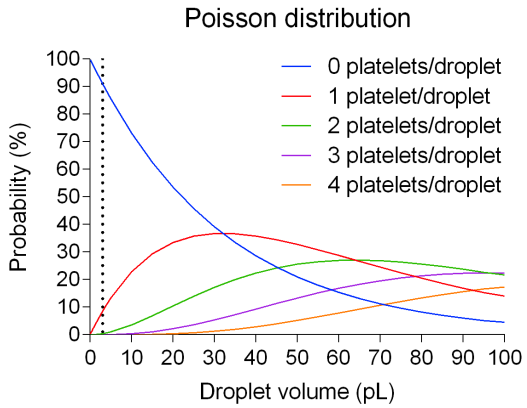


Figure 2-4 Theoretical optimal droplet size determination. Poisson statistic calculated probabilities of 0-4 platelets per droplet. The calculations were made with the assumption of a platelet concentration of 31,250/µL (platelet concentration of 250,000/µL and an 8-fold dilution). At the droplet volume of ~3 pL (vertical black dotted line), 91.1% of the droplets are empty (blue line) and 8.5% contains 1 platelet (red line) while only 0.4% of droplets contains more than one platelet (green line: 2 platelets per droplet, purple line: 3 platelets per droplet and orange line: 4 platelets per droplet).

λ is dependent on both the input platelet concentration and on the droplet size. For this calculation, a platelet concentration of 250×10^9/L (250,000/µL) was assumed, as well as an 8-fold dilution of this platelet sample (i.e. 31,250 platelets/µL). The probabilities of 0-4 platelets per droplet were calculated for droplet sizes ranging 0.1-100 pL (Figure 2-4). An optimal droplet size would result in a reasonably high number of single platelets per droplet without a substantial number of droplets containing more than one platelet per droplet. At a droplet size of ~3 pL (diameter ~18 µm), 91.1% of the droplets are empty and 8.5% contains one platelet while only 0.4% of droplets contains more than one platelet. Therefore, the smallest features of the devices used were 22 µm to accommodate the theoretical optimal droplet size of ~18 µm.

2.3.2 Droplet size and stability is dependent of pinch geometry, total flow rate and flow ratio of aqueous and oil

The formation of droplets in a reliable, stable and monodisperse way is essential for a robust and highly reproducible assay. Several methods have been described in the literature to achieve a stable droplet formation. Initially, four different pinch geometries of the flow focusing junction have been tested (Figure 2-5). These experiments show that a pinch section is needed for the reliable generation of droplets without so-called tip streaming or plug flow formation in which the aqueous compartments are large and elongated by confinement by the channel dimensions.[115, 148] Plug flow is common in the absence of a pinch section, in which the shear forces dictating droplet volume are reduced (Figure 2-5A). A sudden increase in diameter of the outlet channel forms droplets in a monodisperse and stable manner mostly in high flow rates of oil compared to water, but not in lower flow rates where satellite droplets are formed (Figure 2-5B). A gradual increase in diameter of the outlet channel creates droplets at a water to oil ratio of 1:4 but neither

at really high or low ratios does it perform as well as the other geometries (Figure 2-5C). When all inlets are gradually decreasing in diameter combined with a gradual increase of the outlet channel droplets are formed in a monodisperse and stable way at lower oil to water ratios (Figure 2-5D). Because of the increased flexibility in flow rates and oil to water ratios, this design was used for the first part of this study. All initial devices (A-D) had a widening outlet channel and pillar structures at areas of high aspect ratio. The widening outlet channels decrease transport velocity providing more time for the surfactant to appropriately assemble at the 2-phase interface and thus stabilize the droplet. However, droplets merged upon contact with the pillar structures and were therefore removed, by punching the outlet at the site of the first pillar rather than at the end of the channel. For further improvements in design, a combination of the pinch geometries B and D was chosen, because of the stability at high ratios of oil to water of the B geometry and the overall stability of the D geometry (Figure 2-1). This junction resulted in stable, monodisperse droplet formation at both high and low ratios (Figure 2-5E). The pillar structures designed to prevent collapsing of the channel were replaced by filter structures on the oil and agonist inlet and removed from the platelet inlet and outlet. To increase the time in which the surfactant can stabilize the interface the time on chip was lengthened by the addition of a serpentine structure with increased channel width (Figure 2-1E). However, this serpentine structure decreased the distance between the droplets leading to a loss of control over incubation time because of overtaking and occasionally caused collision and coalescence. Therefore, the final design contained a long straight channel to allow sufficient time for the surfactant to stabilize the droplet while not leading to any of those complications (Figure 2-1F). This resulted in stable droplet formation at high and low flow ratios (Figure 2-5F).

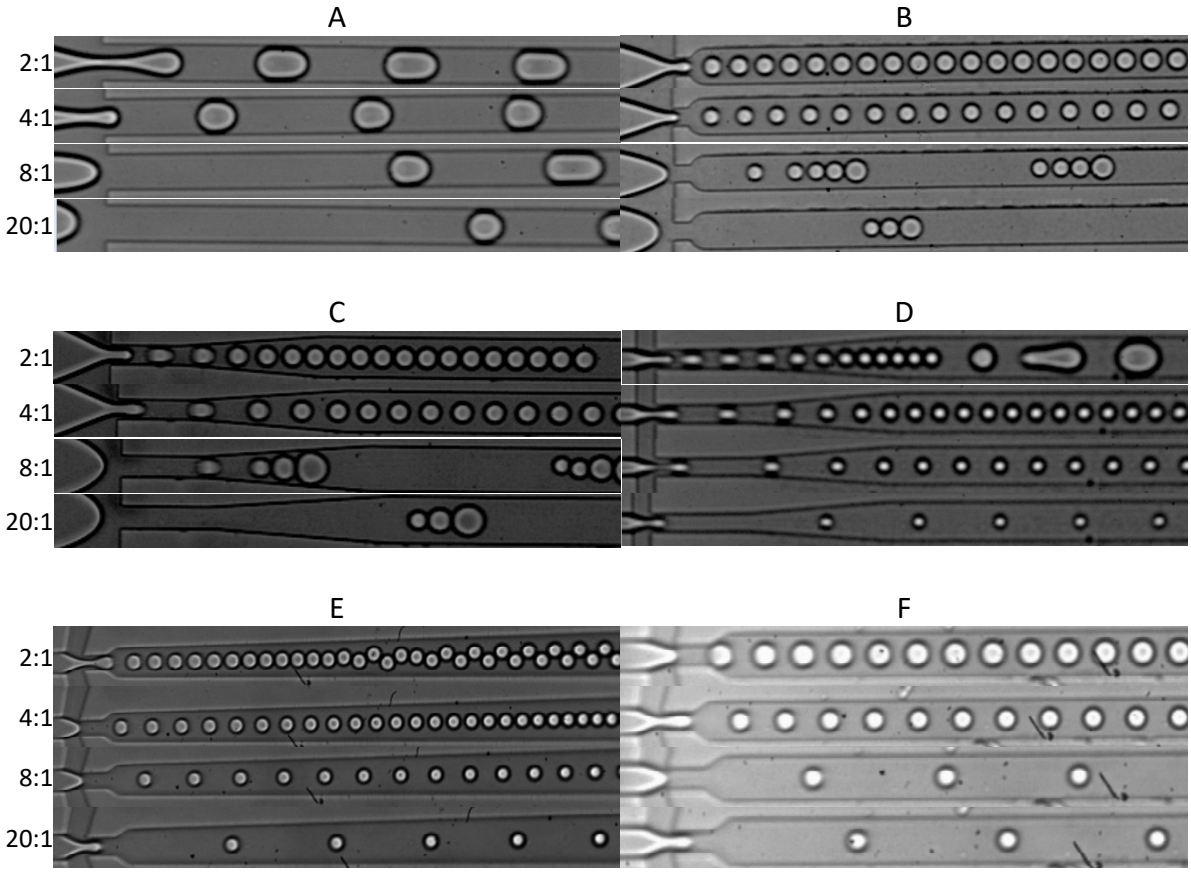


Figure 2-5 The effect of different pinch geometries at various flow conditions. Representative images of live droplet formation at different oil to aqueous ratios using devices with different pinch geometries. Total flow rates are kept constant at 1800 μL/hr (A-D) and 1500 μL/hr (E-F). Number of similar experiments is 3.

A platelet-based assay relies on Poisson statistics to maximize the number of single platelets encapsulated in droplets. The size of the droplet is an important factor in the Poisson equation. Therefore, the size of the droplets needs to be predictable to optimize the encapsulation.

The size of droplets is mostly determined by the dimensions of the channels but can also be modified by changing the flow conditions. These flow conditions are the total flow rate and the ratio of aqueous and oil flows. In order to study the effect of the total flow rate, the total flow rates were varied between 150 and 2500 μL/hr while keeping the ratio of water and oil constant at 1:4. Increasing the flow rate leads to a decrease of the size of the droplets (Figure 2-6). The median of the droplet surface decreases from 477 to 250 μm^2. The variation in the droplet diameter is reviewed with the quartile coefficient of dispersion (QCD, (Q3-Q1)/(Q3+Q1), the non-parametric equivalent for the CV) because the Shapiro-Wilk test was significantly different from a normal distribution. For all total flow rates, the QCD was 5% or lower and most around 2%. An optimal condition would provide stable droplet generation and relatively insensitive to small fluctuations of flow. All rates greater than 1000 μL/hr but smaller than 2000 μL/hr qualify based on these criteria. However, lower flow rates are more cost-effective (as it uses less oil and reagents) while higher are more easily stabilized. Therefore, future work was done with a total rate of 1500 μL/hr.

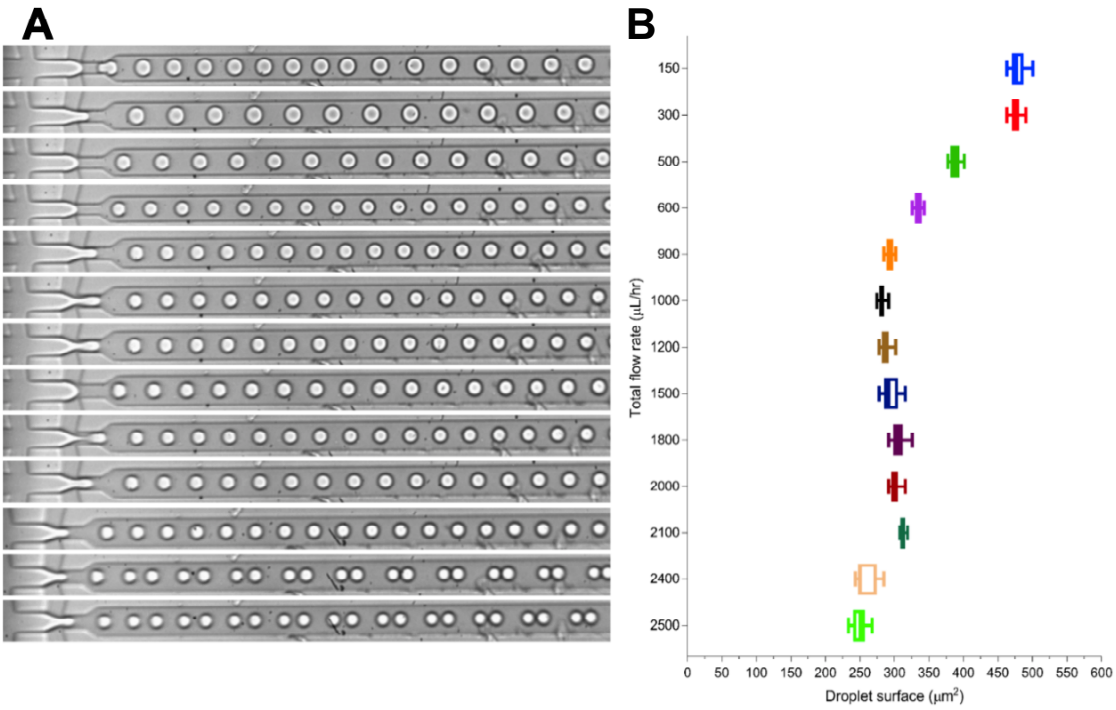


Figure 2-6 The effect of changing the total flow rate on the size of the droplets. The total flow rate of droplet formation was varied between 150 and 2500 µL/hr while keeping the oil to aqueous ratio equal at 1:4. **A)** representative images of live droplet formation, **B)** box and whisker plot of droplet sizes, whiskers represent 10th and 90th percentile. Droplet surface determined with image analysis of live droplet videos using ImageJ. Number of similar experiments is 4. Statistical analysis comprised of non-parametric univariate analysis, median with interquartile range and QCD. QCD for all flow rates was <5.

Another factor that influences the droplet size is the ratio of oil and aqueous flow rates. To test this influence, the total flow rate was kept constant at 1500 µL/hr and the ratios of oil to water were varied from 1:1 to 30:1. Increasing the ratio of water to oil decreases the surface of the droplets from 449 µm^2 at a ratio of 1:1 to 189 µm^2 at a ratio of 30:1 (Figure 2-7). All QCD values were below 5%. Again, lower ratios are more cost-effective because of the lower consumption of surfactant and platelet reagents (such as agonists and antibodies). Consistent, stable droplet generation with sufficient distance between the droplets to prevent coalescence occurred with ratios of 1:3 and above. For future work, a water to oil ratio of 1:4 was chosen because of reliability and economy.

Using these flow conditions, the devices generate monodisperse droplets (QCD <5%). However, assuming the droplets are spherical, calculating the measured droplet surface leads to diameters of >22um in some conditions. But the height of the channel is only 20 µm. This limited height prevents the droplets from expanding in all directions equally. So, most likely the droplets are not spherical while in the device and the diameter or volume cannot be inferred from the observed droplet surface.

Changing the flow ratio of the two aqueous inlets can make it possible to dilute the platelet suspension "on chip". Because diluting a platelet suspension can influence their response this is a useful capability. However, keeping the droplet size stable is essential for a reliable encapsulation rate and to make sure that the probability of encapsulating more than one platelet remains low. Therefore, the influence of changing this ratio of the aqueous flow rates on the droplet size was tested (in device Figure 2-1D) by keeping the total flow rate and the ratio of water to oil stable at 1800 µL/hr and 1:4, respectively.

The flow rate of the agonist inlet was increased while the platelet inlet was simultaneously decreased to increase the ratio but keep the total aqueous flow constant. This increased

ratio did not lead to a difference in droplet size (Figure A-3). Furthermore, for all conditions QCD were below 2%. However, the higher ratios take longer to stabilise the droplet generation. Therefore, for future work a ratio of 1:10 was chosen.

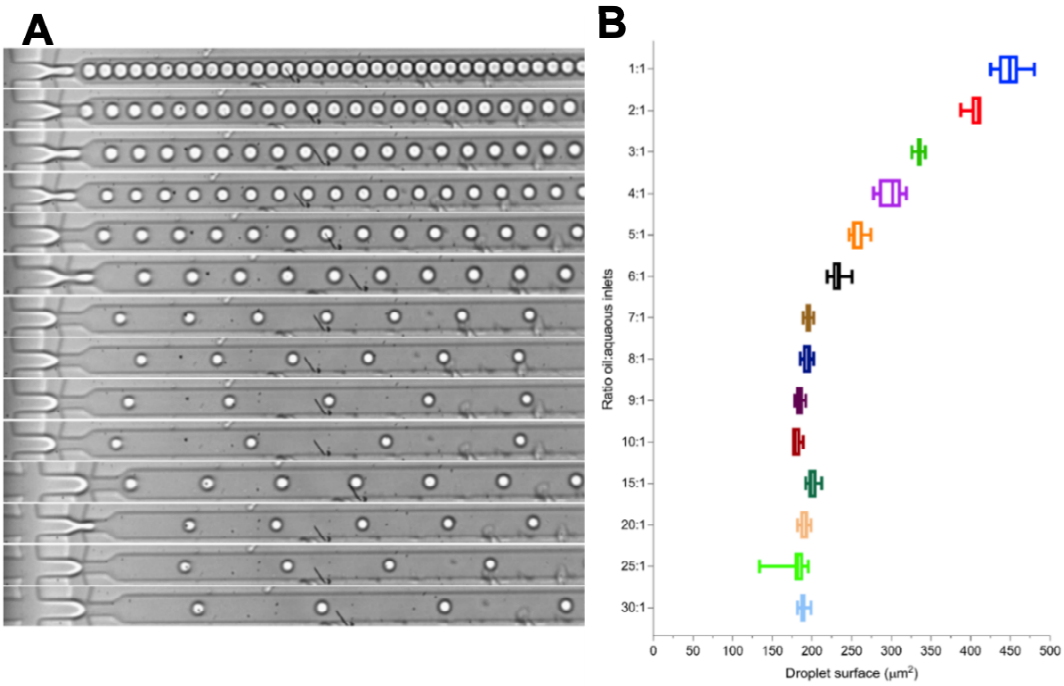


Figure 2-7 The effect of changing the flow ratio of oil to aqueous input on the size of the droplets. The oil to aqueous ratio of droplet formation was varied between 1:1 and 30:1 while keeping the total flow rate equal at 2500 µL/hr. **A)** representative images of live droplet formation, **B)** box and whisker plot of droplet sizes, whiskers represent 10th and 90th percentile. Droplet surface determined with image analysis of live droplet videos using ImageJ. Number of similar experiments is 4. Statistical analysis comprised of non-parametric univariate analysis including QCD. QCD for all flow rates was <5.

2.3.3 Encapsulation of platelet-sized particles follows a Poisson distribution

Encapsulation of cells normally follows a Poisson distribution.[116, 117] The Poisson distribution calculates the encapsulation efficiency using the average number of cells within a droplet which is dependent on the concentration of the particle solution. Subsequently, the probability of encapsulating a defined number of cells within a droplet can be calculated (Figure 2-8).

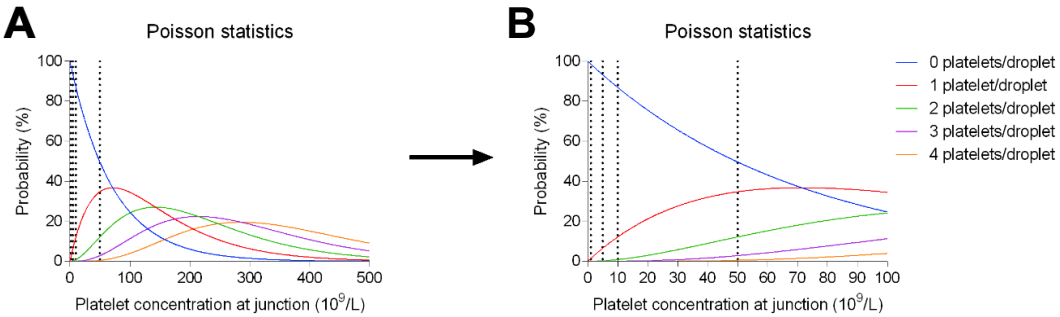


Figure 2-8 Theoretical platelet encapsulation probabilities with various input concentrations of platelets calculated with Poisson statistics. **A)** overview of the relation and **B)** focused on the relevant sections of the relationship. The calculations were made with the assumption of a droplet size of Ø 25 µm ≈ 8 pL (following the optimal input conditions determined in section 2.3.2). Vertical black dotted lines indicate the chosen concentrations for experimental verification at 50, 10, 5 and 1x10⁹/L concentrations. The probability of encapsulating 0 platelets per droplet is shown with a blue line, 1 platelet per droplet with a red line, 2 platelets per droplet with a green line, 3 platelets per droplet with a purple line and 4 platelets per droplet with an orange line.

The correlation between the platelet concentration at the junction and the probability of encapsulating a single platelet shows a strong initial increase and then a gradual decrease. For optimal encapsulation of platelets for the study of single platelet functionality, the probability of encapsulating a single platelet per droplet should be maximal while keeping the probability of encapsulating more than 1 minimal. Theoretically, this indicates a concentration of $10x10^9$/L or lower. To test the correlation between the observed and theoretical platelet encapsulation, platelet-sized particles (Ø 2.1 µm) were encapsulated with 4 concentrations at junction, 50, 10, 5 and $1x10^9$/L (Figure 2-9).

Comparing the observed occupancy with the theoretical value showed a good correlation. However, the number of droplets with no platelet observed is higher than predicted by theory. This could be due to factors not included in the Poisson distribution such as sedimentation of the particles in the syringe, or the presence of particle aggregates (with increased sedimentation rates). Furthermore, the observed encapsulation with $50x10^9$/L is lower for all particles and higher for empty droplets. This would be consistent with a lower platelet concentration, indicating that the assumed droplet size might be too small. With the current droplet size (observed) a concentration of $5x10^9$/L at junction gives a consistently high number of single platelets encapsulated and a low number of more than 1 and is used for future work. With a droplet generating frequency of 500 Hz, approximately 3000/min droplets containing single platelet would be generated. This is sufficiently high to provide a robust statistic of the platelet population and study rare events in a short experimental time. Platelet-sized particles can be encapsulated in droplets and this approximates a Poisson distribution.

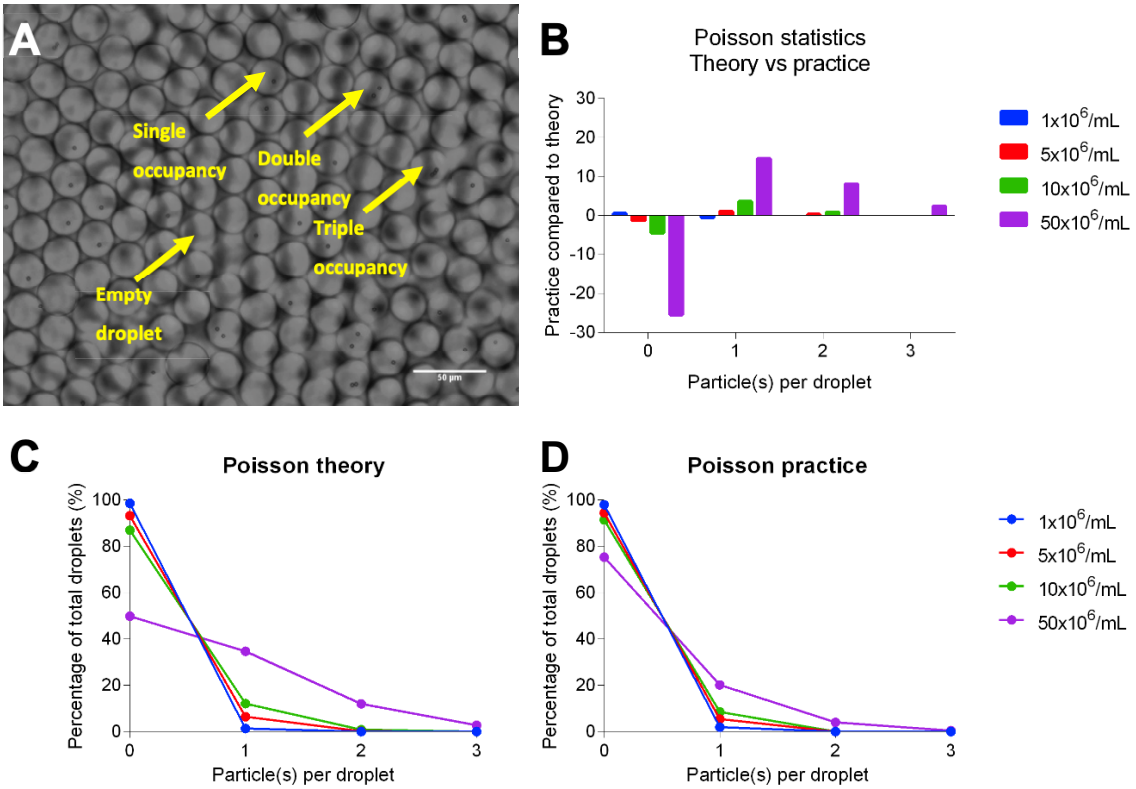


Figure 2-9 The encapsulation of platelet-sized particles in practice compared to the theoretical encapsulation calculated with Poisson statistics (assuming a droplet size of Ø 25 µm ≈ 8 pL). **A)** representative image of platelet-sized (Ø 2.1 µm) particle encapsulation with a particle concentration of $50x10^9$/L, scale bar depicts 50 µm. **B)** the difference between the expected (theoretic calculations) and observed (with microscopy) encapsulation of particles. **C)** the encapsulation in theory and **D)** in practice. Flow conditions as determined optimal in section 2.3.2, device Figure 2-1D, 900 µL/hr total flow rate, oil to aqueous flow ratio 4:1. Number of similar experiments is 2, results shown are from single experiment. Observed encapsulation determined from microscopy images and >100 droplets counted per condition.

A platelet suspension is known to be vulnerable to dilution effects, especially when diluting with autologous platelet poor plasma[149], which tends to decrease the activation capacity of platelets. Furthermore, platelets are only viable up to 4 hours after the blood draw. So, the dilution must not be so high that the increased time needed to get a statistically relevantly number of platelets into droplets is too long to allow several conditions to be studied. In conclusion, the dilution must be high enough to ensure optimal single platelet encapsulation but setting the dilution requires care for dilution effects on activation and the increased time required to run a single condition.

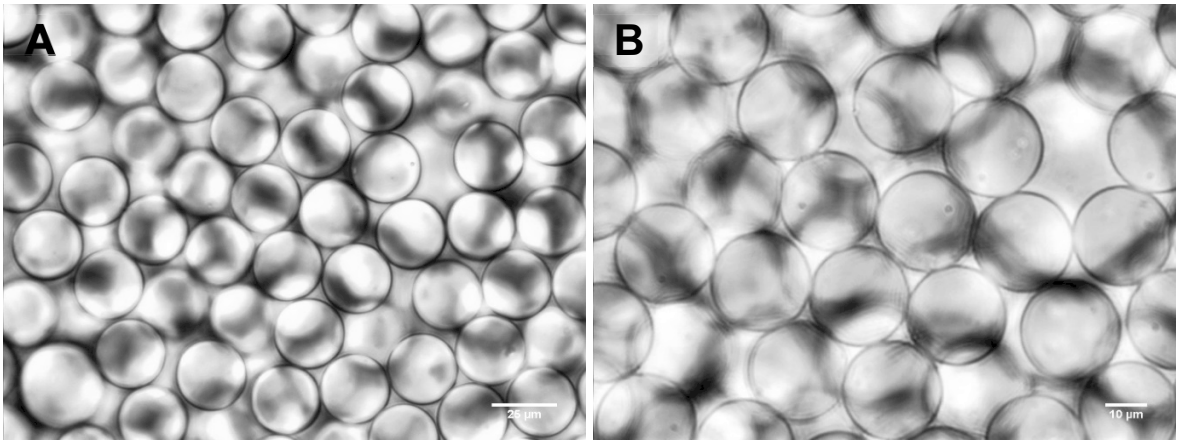


Figure 2-10 Microscopy images of the encapsulation of platelets into droplets. Platelet concentration of $5x10^6$/mL at junction. Scale bars depict left 25 µm and right 10 µm. Flow conditions as determined optimal in section 2.3.2, device Figure 2-1D, 900 µL/hr total flow rate, oil to aqueous flow ratio 4:1. Number of similar experiments is 5.

A platelet concentration of $5x10^6$/mL (=$5x10^9$/L) at the droplet generation junction is shown to be ensuring single platelet encapsulation (Figure 2-10). When using an on chip dilution of 1:10, the platelets are submerged in convulxin and do not require such an extreme dilution off chip. The inlet concentration of platelets needs to be ~$50x10^6$/mL. To limit the dilution effects caused by platelet poor plasma, the platelets are diluted in HEPES buffer instead. With these conditions, collecting droplets for about 10 minutes provides approximately 1000 platelets for cytometry analysis. Later on (from section 2.3.13 onwards) this was changed to 1:5 (inlet concentration of ~$25x10^6$/mL) and a higher total speed of 1500 µL/hr rather than 900 µL/hr leading to quicker stabilisation of flows and ~25000 platelets collected for flow cytometry analysis in 5 min.

2.3.4 A hydrophobic surface treatment of PDMS, required for droplet formation, prevents platelet adhesion and aggregation

Microfluidic droplet formation requires a hydrophobic surface of the channels. However, plasma bonding procedures make the surface hydrophilic. The most common procedure to change this back to hydrophobic is treatment with perfluoro-octyl trichlorosilane (PFTS). To test the effect of this surface treatment a Petri dish pre-coated with PDMS was treated with oxygen plasma, coated with PFTS (1% in HFE-7500) and incubated overnight at 60 °C. For comparison, another Petri dish was untreated (negative control) and one Petri dish was treated with oxygen plasma, coated with collagen, washed and subsequently blocked with 1% BSA (positive control). A platelet suspension was added to all Petri dishes and incubated for an hour. After the hour the Petri dishes were washed with a HEPES buffer solution and photographed by microscopy. No adherent platelets can be observed after treatment with the PFTS and only an occasional platelet can be observed on the untreated PDMS (Figure 2-11). Coating with collagen led to clusters of adhered platelets, indicating an active

adhesion and subsequent activation and aggregation of platelets. Several other surface treatments were tested (Figure A-4). Only on PDMS coated with collagen could clusters of platelets be observed. However, occasional single platelets could be observed in hydrophilic PDMS (after oxygen plasma treatment), including after blocking with 1% BSA. This indicates that the most effective way to prevent passive adherence of platelets can be achieved with a hydrophobic surface treatment, more so than blocking with BSA.

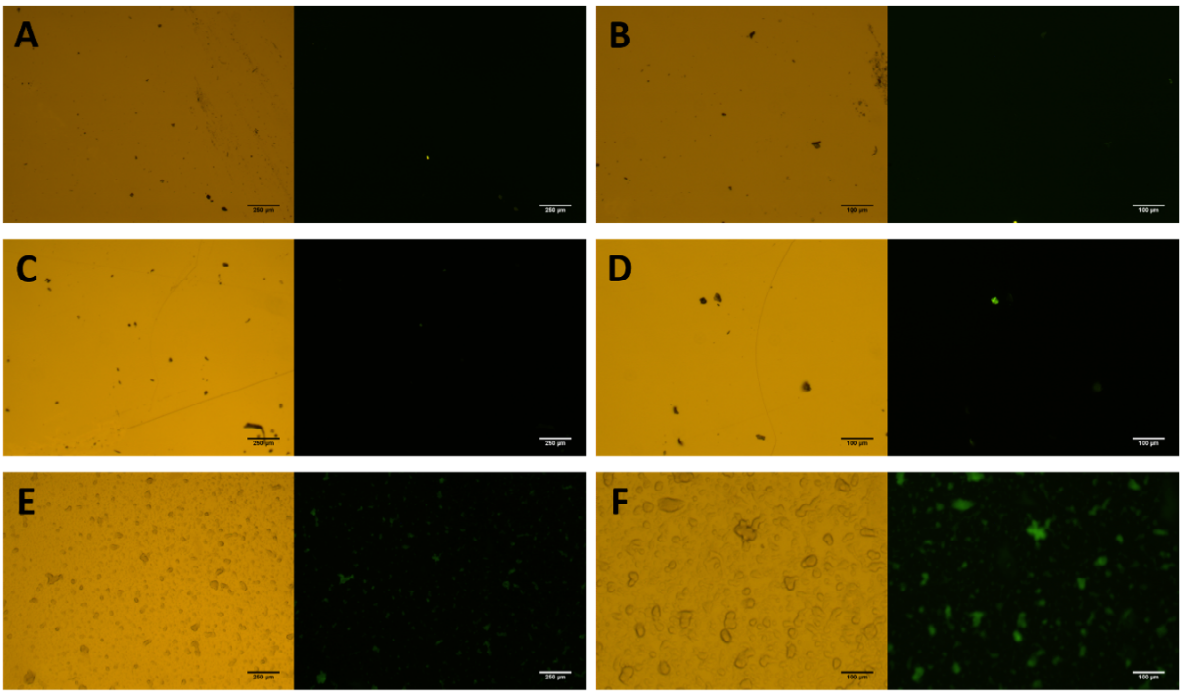


Figure 2-11 Effect of surface treatments of PDMS on platelet adhesion. **A** and **B)** PDMS treated with oxygen plasma, coated with perfluoro-octyl trichlorosilane (PFTS). (1% in HFE-7500) and incubated overnight in a 60 °C oven. **C** and **D)** Untreated PDMS (negative control). **E** and **F)** PDMS treated with oxygen plasma, coated with collagen, washed, blocked with 1% BSA (positive control). **A**, **C** and **E** microscopy image of brightfield and FITC with 4x magnification, scale bar depicts 250 µm. **B**, **D** and **F** zoomed in with 10x magnification, scale bar depicts 100 µm. Platelet rich plasma stained with $DiOC_6$. Number of similar experiments is 2.

2.3.5 Optimal platelet gating combines a platelet label, forward-sideward scatter and doublet-exclusion gates

A platelet gate set purely on forward and sideward scatter is prone to contamination by other small particles.[78, 133] Furthermore, doublets and swarming are other potential problems, were more than one particle passes the detector simultaneously. This is a bigger problem when the particles of interest are smaller than standard cells such as platelets. Dilution can partially prevent this, but some swarming can remain which can influence the results. For this study it was decided to gate platelets with a specific platelet label (anti-CD42b in most cases and otherwise with anti-CD61) and gate out events outside of the forward and sideward gate for platelets as well as gating out any doublets (Figure 2-12). The gates were set using the 8 donor data discussed in chapter 3. The platelet gate was selected using Flowjo and subsequently for regular intervals the minimum and maximum of the gate were determined. This gate was subsequently tested on all 8 donor data individually and on the other samples discussed in chapter 3. An example of this test can be found in Figure 2-13 for on chip data and Figure 2-14 for off chip data. The number of doublets observed in off chip samples is higher compared to on chip samples providing additional evidence for the single platelet encapsulation in the droplets. Because the

activity gate was not affected and only intended events were excluded this gate was applied for all samples in the next chapters.

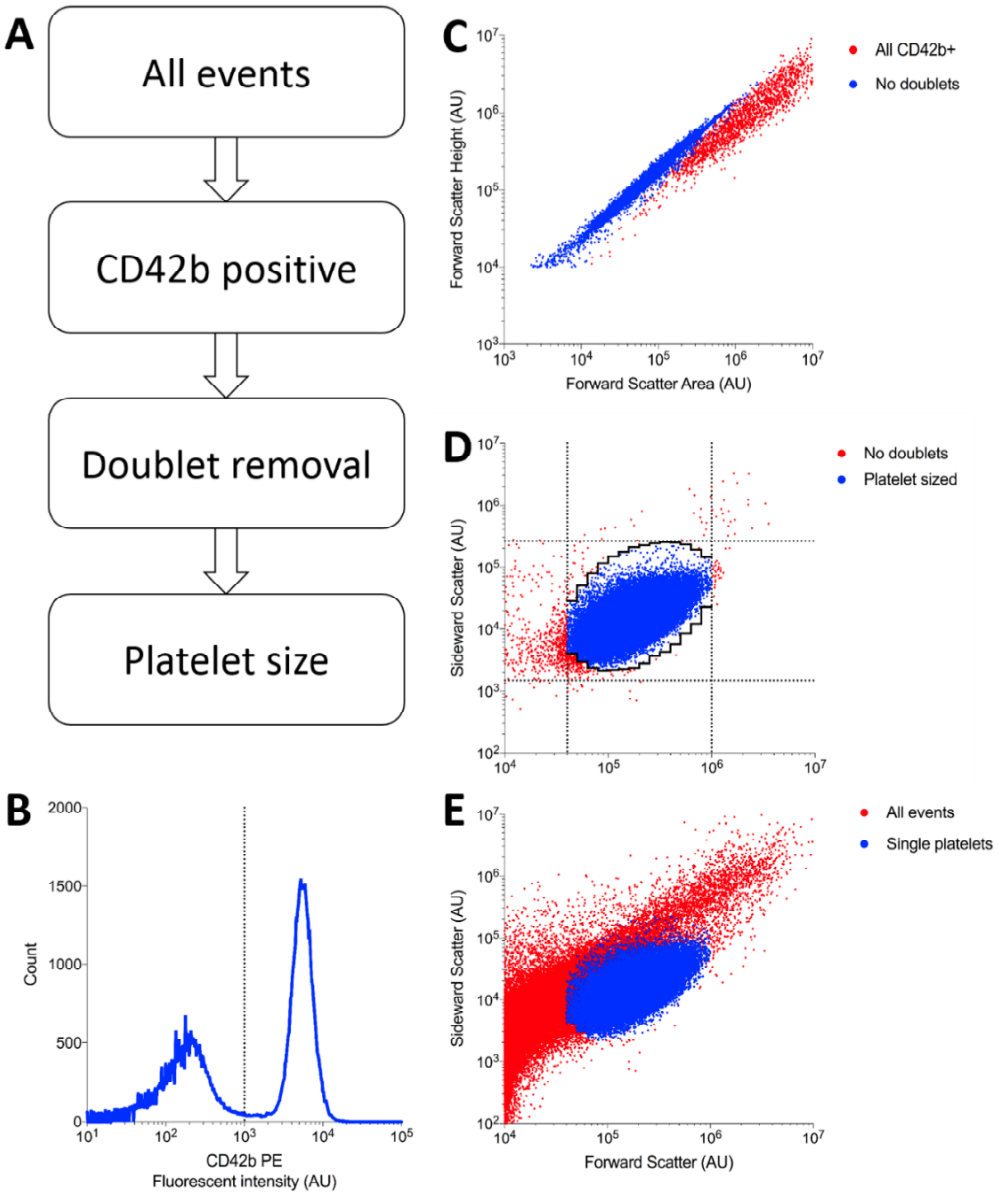


Figure 2-12 Gating strategy for selecting platelets from background noise and contamination. **A)** the steps involved in the gating process. Graphical representation of gating strategy: **B)** gating based on CD42b signal. **C)** a doublet and swarm removal gate. **D)** gating based on forward- and sideward-scatter. **E)** overall effect of gating (raw data in red). Scatterplots C-E show original gate in red and applied gate in blue.

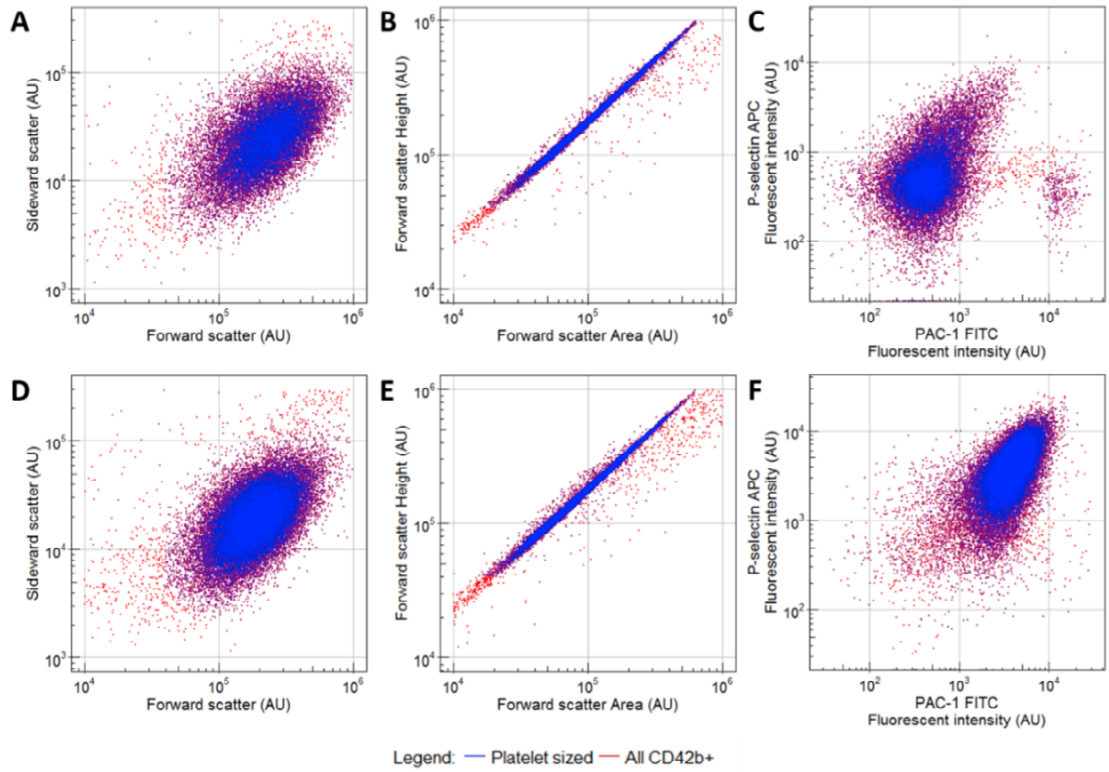


Figure 2-13 On chip example of the effect of platelet gating strategy used. Events within the platelet gate are shown in blue and all events positive for the platelet specific antibody CD42b are shown in red. **A)** Forward and sideward scatter gate. **B)** the doublet exclusion gate with the area vs height of forward scatter signal. **C)** the effect of the gate on the platelet activity of both PAC-1 and P-selectin signal. **D-F)** same for sample stimulated with 100 ng/mL of convulxin.

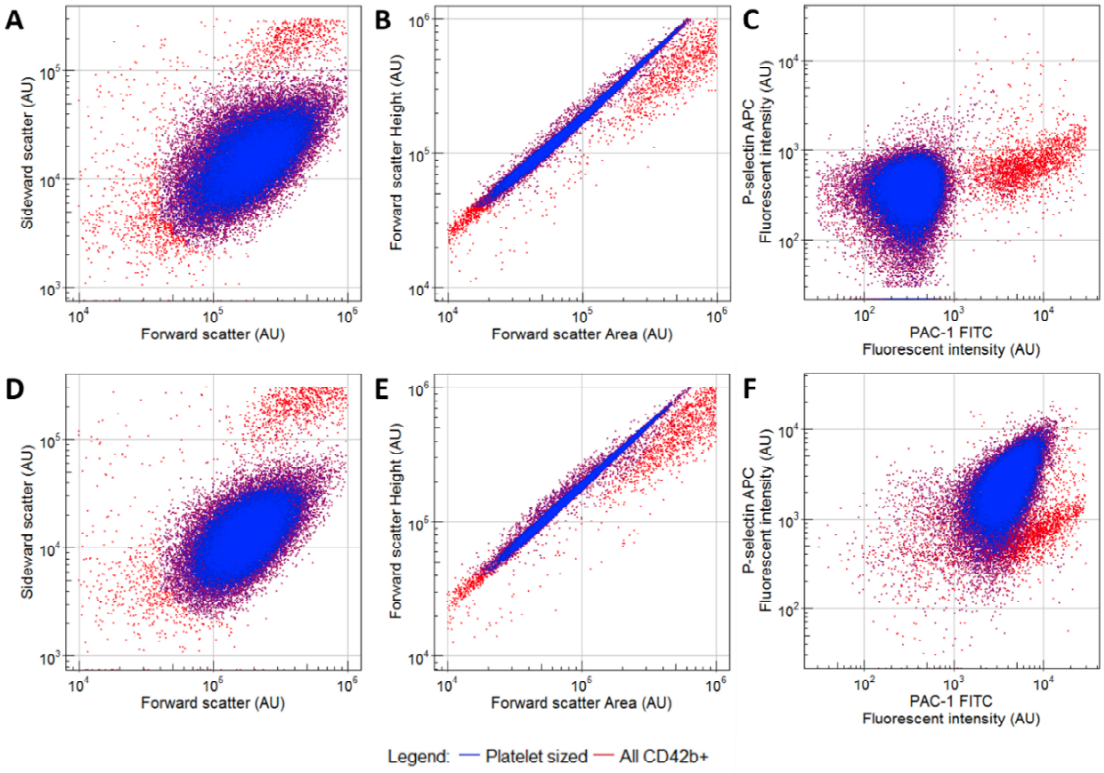


Figure 2-14 Off chip example of the effect of platelet gating strategy used. Events within the platelet gate are shown in blue and all events positive for the platelet specific antibody CD42b are shown in red. **A)** Forward and sideward scatter gate. **B)** the doublet exclusion gate with the area vs height of forward scatter signal. **C)** the effect of the gate on the platelet activity of both PAC-1 and P-selectin signal. **D-F)** same for sample stimulated with 100 ng/mL of convulxin.

2.3.6 Antibody titration is required for optimization of signal to noise

Antibody concentrations need to be optimized to increase signal to noise ratio, make the assays cost-effective and decrease the effect of non-specific binding. In order to find the optimal concentration of PAC-1 antibody, specifically targeting the active conformation of the $\alpha_{IIb}\beta_3$ receptor of platelets, the concentration of PAC-1 antibody was titrated with a serial dilution (concentrations of 0.1, 0.2, 0.39, 0.78, 1.56 3.13 and 6.25 ng/µL). Samples treated with 100 ng/mL convulxin (activating the platelets) were compared with the vehicle (HEPES buffer in which the convulxin was diluted) and the isotype control at the same concentrations. The median fluorescence intensity (MFI) is calculated from events in the forward and sideward scatter based platelet gate (for an example see section 2.3.5 and Figure 2-12D). This MFI was plotted against the concentration of PAC-1 antibody on a log-scale, as median with quartiles (Figure 2-15A).

The mean fluorescence intensity of the convulxin treated platelets shows a clear separation (ratio > 10) from the vehicle control from concentrations of 0.39 ng/µL PAC-1 antibody and upwards (Figure 2-15A). At higher concentrations of antibody, untreated platelets show an increased signal compared to lower concentrations, decreasing the signal to noise. The choice of the optimal concentration also depended on cost-effectiveness. The optimal concentration of the PAC-1 antibody was determined as 0.78 ng/µL. This concentration shows a clear distinction between stimulated and unstimulated platelets and caused no increased signal in both the vehicle and isotype controls, while also being cost-effective (Figure 2-15A).

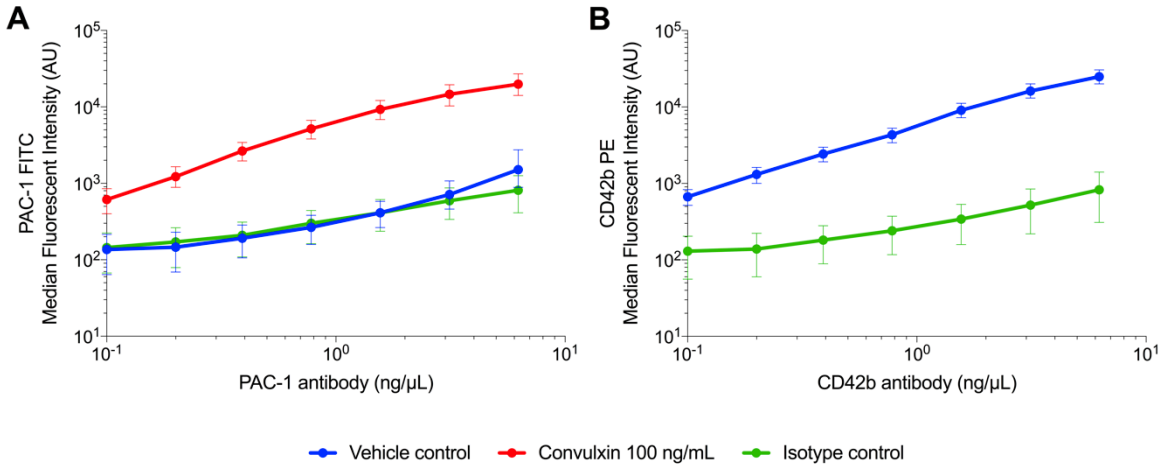


Figure 2-15 Median fluorescence intensity of antibody titration of flow cytometry samples activated with 100 ng/mL convulxin (red line) compared to the vehicle (blue line) and isotype control (green line). Titration shown of **A)** PAC-1 antibody and **B)** CD42b antibody. The MFI is obtained from the forward and sideward scatter gate, with median intensity plotted with quartiles. N=3 Statistical analysis comprised of non-parametric univariate analysis of single platelets.

An antibody that can be used to identify platelets is anti-CD42b which targets a ubiquitously expressed, platelet specific receptor, the GPIb tail of the GPIb-V-IX receptor complex. This receptor complex is involved in the adherence of platelets to von Willebrand factor. The anti-CD42b was titrated in whole blood, with a serial dilution (concentrations of 0.1, 0.2, 0.39, 0.78, 1.56 3.13 and 6.25 ng/µL), resulting in a clear separation of the antibody and the isotype control (Figure 2-15B). The median of fluorescence intensity (MFI) is calculated from events in the forward and sideward scatter based platelet gate. This median with quartiles was plotted against the concentration of anti-CD42b antibody on a log-scale. Good discrimination of the antibody and isotype control is observed at 0.39 ng/µL or above. For further experiments a concentration of 0.78 ng/µL was chosen because of the clear

distinction between antibody and isotype control, the low bleed through into other channels and being cost-effective (Figure 2-15B). Two other antibodies were titrated at this stage, anti-CD61 and P-selectin, and can be found in the appendix (Figure A-5).

However, changes in assay conditions such as whole blood compared to platelet rich plasma can possibly affect the optimal concentration of antibody. Furthermore, new batches of antibody require verification. Platelet pre-incubation and encapsulation of platelets with antibody (to be discussed later see section 2.3.10) were considerably less sensitive to antibody binding and required higher concentrations. Therefore, chosen concentrations were adapted and titrations repeated where necessary.

2.3.7 Selected antibody panels are suitable for multi-colour acquisition without compensation

Multi-colour acquisition of signals on a cytometer is a big advantage of using flow cytometry but is susceptible to bleed through effects of one fluorochrome into the acquisition channel of another fluorochrome because of partially overlapping emission spectra. This problem can be partly solved by compensation. The bleed through can be quantified with a fluorescent minus one (FMO) control, where antibodies are left out one by one.

The results show that there was minimal bleed through into channels 1 and 4 (Figure 2-16A and C). The resultant signal was around the same level as auto-fluorescence and no difference was observed between active and non-active platelets. There was a higher signal than expected in channel 2 when the anti-CD42b was left out (Figure 2-16B). This is most likely due to bleed through from the FITC conjugated PAC-1 antibody. However, the increased signal is not high enough to interfere with appropriate distinction between a platelet and other particles. Furthermore, the $\alpha_{IIb}\beta_3$ receptor is platelet specific meaning a particle cannot be positive for PAC-1 and negative for CD42b. Therefore, compensation was determined to be unnecessary. Other combinations (antibody panels) can be found in the appendix (Figure A-6), as well as the raw data scatter plots (Figure A-7, Figure A-8, Figure A-9 and Figure A-10).

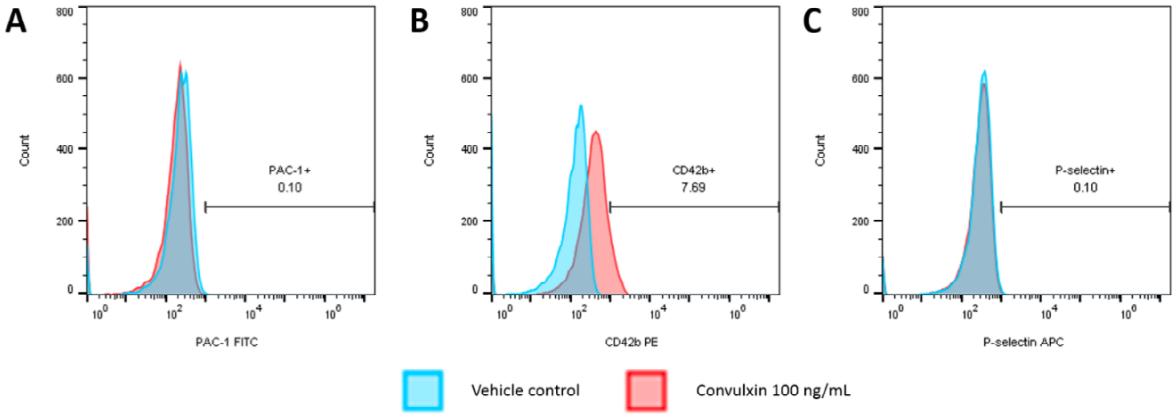


Figure 2-16 A fluorescence minus one control for the simultaneous use of FITC conjugated PAC-1, PE conjugated anti-CD42b and APC conjugated anti-P-selectin. **A)** The mean fluorescence intensity of events within channel 1 (533/30) without PAC-1. The only expected signal in this channel is from the PAC-1 antibody. **B)** Observed MFI within channel 2 (585/40) without anti-CD42b, the expected signal from anti-CD42b. **C)** Observed MFI in channel 4 (675/25) without anti-P-selectin, expected signal in this channel comes from anti-P-selectin. Comparing bleed through and background of platelets stimulated with vehicle in blue and 100 ng/mL convulxin in red. Events shown based on a forward and sideward scatter platelet gate. N=2.

Using CD61 as a target for the identification of platelets is standard but presents with a potential problem when used in conjunction with PAC-1. While PAC-1 only targets the active $\alpha_{IIb}\beta_3$ receptor (fibrinogen binding site), anti-CD61 targets the β_3 tail. Therefore, it could be that anti-CD61 interferes with binding of PAC-1 and PAC-1 with anti-CD61 binding. To test if this interference is present in this assay, the two antibodies were tested simultaneously and compared with allowing PAC-1 to bind first and adding CD61 after sufficient incubation time. This experiment shows that the signal observed for PAC-1 binding is almost twice as high when used sequentially rather than simultaneously (Figure 2-17A). Also, CD61 is lower when added sequentially indicating that anti-CD61 binding is a cause for the reduced PAC-1 binding (Figure 2-17B). No difference in unstimulated PAC-1 binding is observed. Stimulation with convulxin has the effect to increase anti-CD61 binding. This is expected because degranulation of activated platelets causes the membrane of the granules to fuse with the plasma membrane and deliver additional $\alpha_{IIb}\beta_3$ receptor copies. Because sequential staining is not an option within the droplets panels were designed to contain either PAC-1 or anti-CD61 and use anti-CD42b as an alternative for anti-CD61.

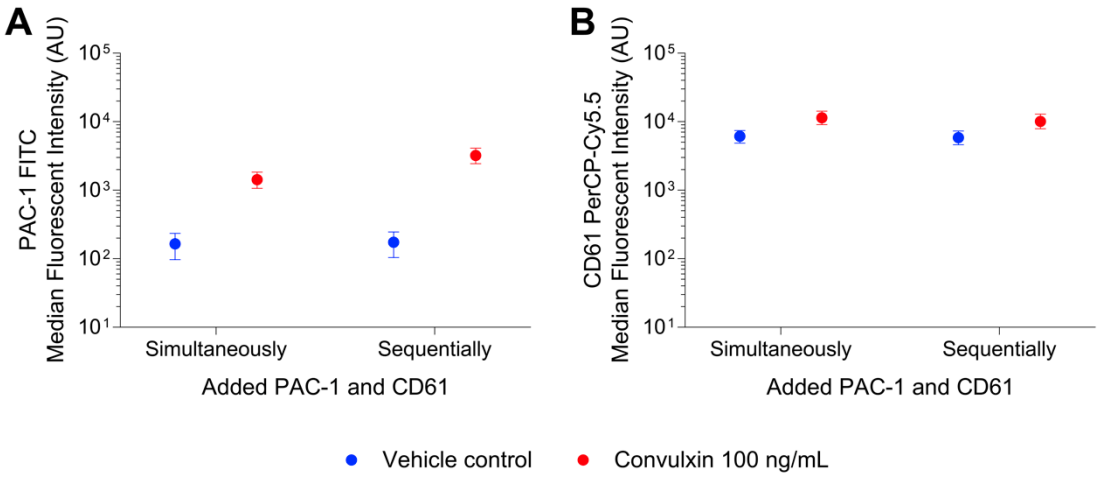


Figure 2-17 The effect of anti-CD61 on PAC-1 binding on stimulated (with 100 ng/mL convulxin in red) and non-stimulated platelets (vehicle control in blue). PAC-1 was added first and given time to bind before anti-CD61 was added to the samples (sequentially). This was compared to adding both antibodies simultaneously. **A)** The median fluorescent intensity of PAC-1 signal and **B)** of anti-CD61 signal. Median fluorescent intensity with quartiles shown from events in a platelet gate based on forward and sideward scatter. A minimum of 10000 events within the platelet gate was measured for each condition. Number of independent experiments is 1. Statistical analysis comprised of non-parametric univariate analysis of single platelets.

2.3.8 Rivaroxaban is suitable for preventing platelet activation by CaCl2

In order to study the phosphatidylserine (PS) exposure on the surface of platelets, this study used Annexin V. Annexin V binding is one of the most studied sources of heterogeneity of platelets and represents the pro-coagulant phenotype.[59] Both PS exposure and Annexin V require a medium containing $CaCl_2$. However, the anti-coagulant used in this study, 3.2% citrate, inhibits coagulation by removing free $CaCl_2$ and is fully reversible by addition of $CaCl_2$ to the plasma.[150] Furthermore, the thrombin formed by the coagulation cascade is a strong platelet agonist. This makes it difficult to study activation in response to a specific stimulus. To prevent thrombin formation by the coagulation cascade but without inhibiting thrombin itself, so that the possibility remains to use added thrombin

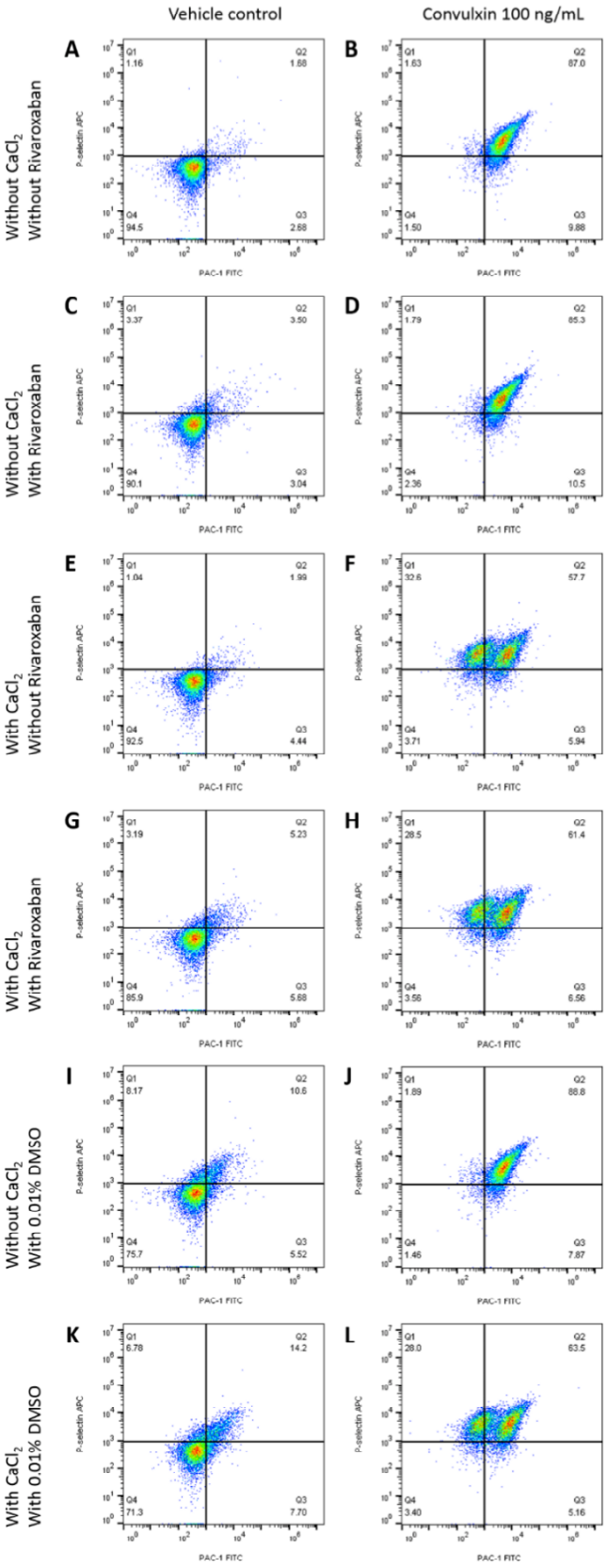


Figure 2-18 The effect of Rivaroxaban (0.1 μM) on platelet activation caused by $CaCl_2$. Platelets were incubated in buffer with or without Rivaroxaban for 15 min. Subsequently, this platelet suspension was added to agonist and antibody solution with or without added $CaCl_2$ (or 0.01% DMSO). PAC-1 and P-selectin activity of platelets in the **A** and **B)** absence of $CaCl_2$ or Rivaroxaban. **C** and **D)** presence of Rivaroxaban and absence of $CaCl_2$. **E** and **F)** presence of $CaCl_2$ and absence of Rivaroxaban. **G** and **H)** presence of both $CaCl_2$ and Rivaroxaban. **I** and **J)** presence of 0.01% DMSO (the medium in which the Rivaroxaban was dissolved) and absence of $CaCl_2$. **K** and **L)** presence of 0.01% DMSO and $CaCl_2$. Platelets in **A, C, E, G, I, K** were not activated (vehicle control) while platelets in **B, D, F, H, J** and **L** were activated with 100 ng/mL of convulxin. Number of independent experiments is 1.

as an agonist, this study applies the specific factor Xa inhibitor Rivaroxaban.[151-153] While this compound is widely used in clinical applications it is new in platelet assays. This study verified the effects on platelet function even though no effects are expected. To this effect, PRP was diluted with buffer to $50x10^9$/L with HEPES buffer and subsequently diluted 1:1 in a solution of HEPES buffer with or without 0.1 μM Rivaroxaban. After incubation of 15 min it was added to agonist and antibody solution with or without added $CaCl_2$ (Figure 2-18). This shows that Rivaroxaban does not inhibit the activation by convulxin and can be used to prevent the platelet activation resulting from thrombin formation (Figure 2-18B compared to D and F compared to H). While no activation is observed as a result of addition of $CaCl_2$ in this case (Figure 2-18E), exclusion of this route of platelet activation, by addition of Rivaroxaban, increases the specificity of the assay. Furthermore, the addition of 0.01% DMSO has no influence on the platelet activity measured with PAC-1 and anti-P-selectin. Other antibody panels showed a similar effect of Rivaroxaban (Figure A-11 and Figure A-12).

2.3.9 Fixative preserves existing antibody binding but interferes with new antibody binding on the surface of platelets

Fixation of samples provides flexibility in measuring activation when a cytometer is not readily available. Furthermore, when releasing platelets from the droplets, platelets may be exposed to conditions that could lead to unintentional activation. Fixative would prevent this unintentional *post factum* activation. To estimate how well fixative prevents loss of staining, both stimulated and unstimulated samples were fixed and subsequently measured every day for a total of 5 days after activation and fixation (Figure 2-19). This was compared to samples diluted with HEPES buffer. Directly after activation there is a small effect of fixation on the amount of staining measured by cytometry; the magnitude of activation is lower with fixative. The consistency of non-fixed samples was difficult to interpret because the platelets did not retain their size (for forward and sideward scatter gating) nor their identification label. However, of those that could be measured, the signal in activation antibodies increased within 4 hours after activation. On the other hand, the fixed samples differed a bit between 0 and 4 hours after activation (slight reduction in P-selectin signal) but remained stable after that for at least 4 days. All future samples were therefore fixed and subsequently measured within 4 days.

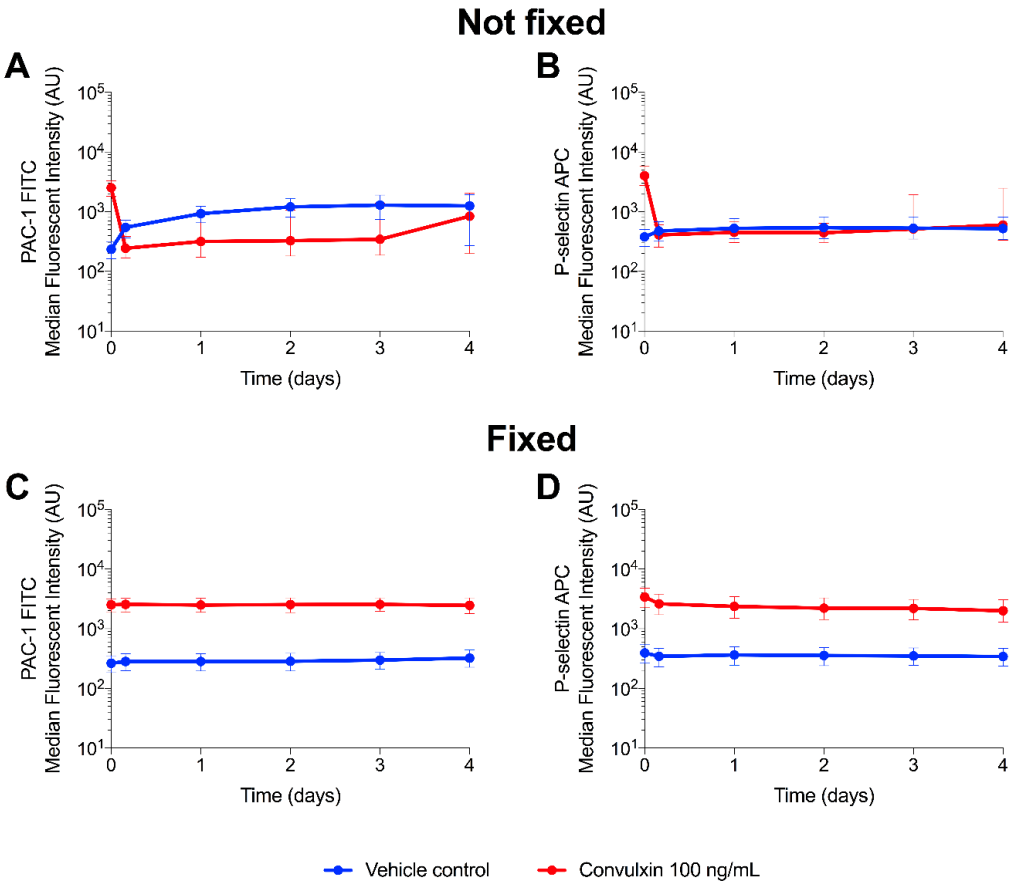


Figure 2-19 The measurement of the effect of fixation on the preservation of staining on both stimulated (red line) and unstimulated platelets (blue line). Samples were repeatedly measured at 0 and 4 hours, and day 2-5. **A)** The preservation of staining of PAC-1, and **B)** anti-CD62P without fixation (only diluted with HEPES buffer). **C)** The preservation of staining of PAC-1, and **D)** anti-CD62P after fixation with BD CellFix™. Median fluorescent intensity with quartiles shown from events in a platelet gate based on forward and sideward scatter. A minimum of 10000 events within the platelet gate was measured for each condition. Number of independent experiments is 1. Statistical analysis comprised of non-parametric univariate analysis of single platelets.

While using fixative has many advantages, it is known that it limits the binding of antibodies, especially of PAC-1[78]. To test the effect fixative has on the binding of the antibodies used in this study, a comparison was made between samples fixed before and after staining (Figure 2-20). This showed the earlier described decrease in intensity when fixed after staining. This is fairly small and does not interfere with an accurate distinction as active or non-active. The expected limitation of PAC-1 binding to (already) fixed samples is observed and is also observed for anti-CD61 and anti-CD62P. The limitation of antibody binding is too large to make an accurate distinction between active and non-active platelets and identification of platelets is also not possible. Therefore, it is necessary to add the antibodies followed by an incubation time for (optimal) binding before fixing the samples. This means that in the droplet system antibodies need to be added to before encapsulation in droplets because the platelets are removed from the droplets into fixative.

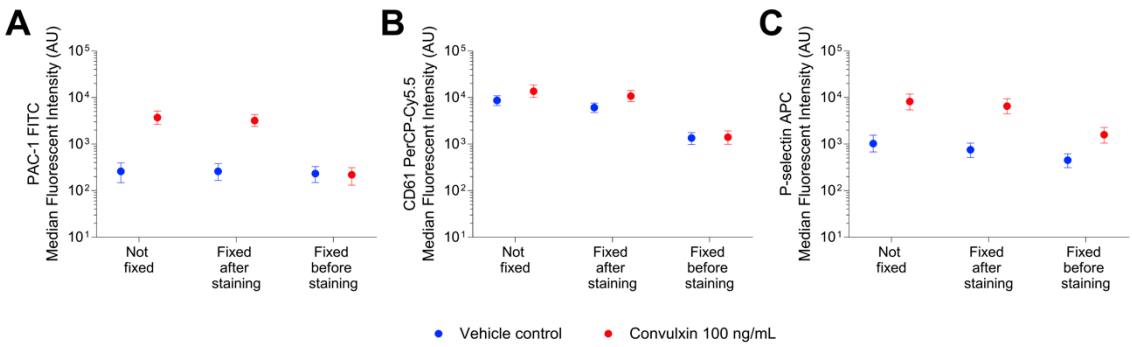


Figure 2-20 The effect of fixative on binding of antibodies. Samples are either non-fixed, fixed after antibody binding or fixed before antibody binding. The effects of fixative on binding with **A)** PAC-1, **B)** anti-CD61 and **C)** anti-CD62P are measured with both stimulated samples (100 ng/mL of convulxin, red) and vehicle controls (blue). Procedures were similar for all 3 conditions. Median fluorescent intensity with quartiles shown from events in a platelet gate based on forward and sideward scatter. A minimum of 10000 events within the platelet gate was measured for each condition. Number of independent experiments is 1. Statistical analysis comprised of non-parametric univariate analysis of single platelets.

2.3.10 Platelets can be pre-incubated with antibodies prior to encapsulation in droplets

Non-specific binding of antibodies is a potential problem in all antibody based assays, especially those without washing steps. Therefore, titrating the antibody is essential for a high quality assay. For the droplet assay and using a 3-inlet device (Figure 2-1), the platelets need to be incubated with antibodies in diluted PRP before activation (pre-incubated PRP) and so this is more susceptible to non-specific binding also because other cells that can have similar sensitivity to non-specific binding are removed (such as red blood cells). However, this pre-incubated PRP is more difficult to work with in terms of a titration. The changes in this assay make conversion of concentrations more difficult. Therefore, the optimal concentrations of the standard whole blood flow cytometry assay (see section 2.3.6) were theoretically converted for the pre-incubated PRP method and multiple concentrations of antibody close to this theoretical optimal checked. One additional problem is that the addition of antibodies straight into the platelet suspension, seemed to cause activation (Figure A-13). Diluting the platelet suspension 1:1 in a solution of antibodies diluted in HEPES buffer solved this problem. Antibody solutions with a final concentration (after the 1:1 dilution into the platelet suspension) of 0.94, 1.25, 1.56, 1.88, 2.5 and 3.75 ng/µL of PAC-1 antibody, 0.47, 0.63, 0.78, 0.94, 1.25 and 1.88 ng/µL P-selectin antibody and 0.94, 1.25, 1.56, 1.88, 2.5 and 3.75 ng/µL of CD42b antibody were tested (Figure 2-21). The overall signal to noise was reduced compared to the whole blood standard assay (Figure 2-15 and Figure A-5) as expected due to the increased non-specific binding. However, a clear distinction between non-active (vehicle control), medium activation (1 ng/mL convulxin) and full activation (100 ng/mL convulxin) can be made with concentrations of 1.25, 0.63 and 1.25 ng/µL of PAC-1, anti-P-selectin and anti-CD42b respectively, while still being relatively cost-effective. Therefore, the incubation of PRP with antibodies prior to activation is an effective staining routine for single platelet function measurements within a droplet analytical pipeline.

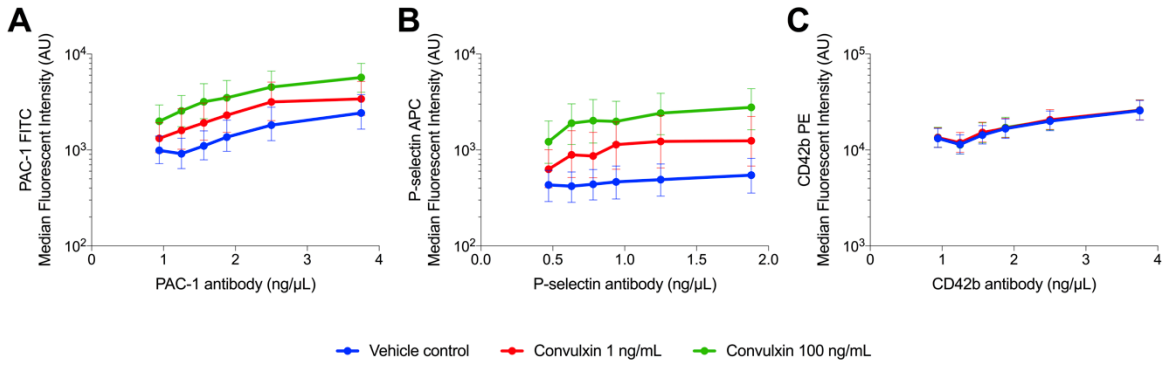


Figure 2-21 Median fluorescence intensity of antibody titration of pre-incubated PRP samples activated with 100 ng/mL convulxin (green) compared to 1 ng/mL (red) and the vehicle (blue). PRP was added to an antibody solution (in a 1:1 ratio), incubated for min 15 min before addition to diluted agonist. **A)** PAC-1 antibody, **B)** P-selectin antibody and **C)** CD42b antibody. The MFI is obtained from the forward and sideward scatter gate, with median intensity plotted with quartiles. A minimum of 10000 events within the platelet gate was measured for each condition. Number of independent experiments is 1. Statistical analysis comprised of non-parametric univariate analysis of single platelets.

2.3.11 Platelets can be encapsulated, activated and stained within droplets before retrieving them from droplets

Platelets pre-incubated with antibodies were added to a syringe and transported through a device where they were encapsulated in the presence of (different concentrations of) convulxin and a vehicle control condition. Comparing this with the same PRP activated off chip estimates the effect of the encapsulation and the handling needed before and after encapsulation. Platelets that were encapsulated in droplets had an increase in both integrin $\alpha_{IIb}\beta_3$ activation and P-selectin expression from the vehicle control to the 10 ng/mL of convulxin (Figure 2-22 and Table A-1). The $\alpha_{IIb}\beta_3$ activation can be observed from a concentration of 0.1 ng/mL and onwards (Figure 2-22A) while the P-selectin is only observed at a concentration of 10 ng/mL (Figure 2-22B). However, there seems to be some activation of the negative control present, although this seems more related to the pre-incubation with antibodies than the encapsulation in droplets because it is also observed in the off chip control (Figure A-14). Furthermore, when comparing antibodies with isotype control within the droplets it seems that for the PAC-1 binding the main source of the decreased signal to noise is an increase in binding to the vehicle control while the anti-P-selectin seems to be more related to an increase in non-specific binding (Figure 2-23 and Table A-2).

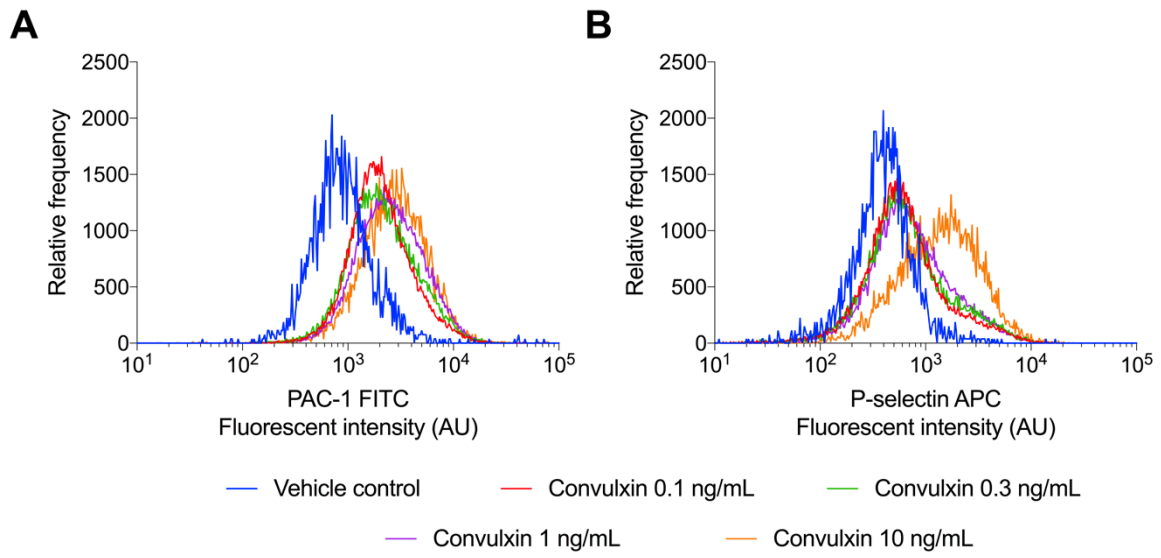


Figure 2-22 Dose response relationship of single platelet activity (pre-incubated with antibodies and in droplets (on chip, in device)) in response to convulxin. Platelets were stimulated with 0.1 ng/mL (red), 0.3 ng/mL (green), 1 ng/mL (purple), 10 ng/mL (orange) and non-stimulated (vehicle control, blue). After incubation in droplets the emulsion was broken and antibody binding measured on a flow cytometer. Platelet activity measured with **A)** PAC-1 antibody and **B)** P-selectin antibody. N=1 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table A-1. PAC-1 binding had RA>2 for all convulxin treatments while P-selectin binding only 10 ng/mL convulxin had RA>2.

To test the effects of retrieval of platelets from droplets, three different methods of breaking the emulsion were tested. Firstly, the droplets were collected and incubated in a tube on the bench, then fixative and subsequently perfluoroctanol (PFO) was added, and the platelets retrieved (indirect breaking). Secondly, very similar procedure but with the addition of a layer of mineral oil on top of the emulsion to protect the droplets during incubation (indirect breaking with mineral oil). Finally, the droplets were incubated within a long piece of outlet tubing and collected directly into a tube where fixative and PFO were already present (direct breaking). Signal to noise was similar with indirect breaking with or without mineral oil but reduced with direct breaking (Figure 2-24 and Table A-3). However, none of the breaking methods results in a signal to noise comparable to off chip control. In conclusion, the pre-incubation, encapsulation and removal from droplets are feasible and a measurable activation level is observed when activating the platelets within droplets with convulxin. However, optimizing the signal to noise further would improve the assay.

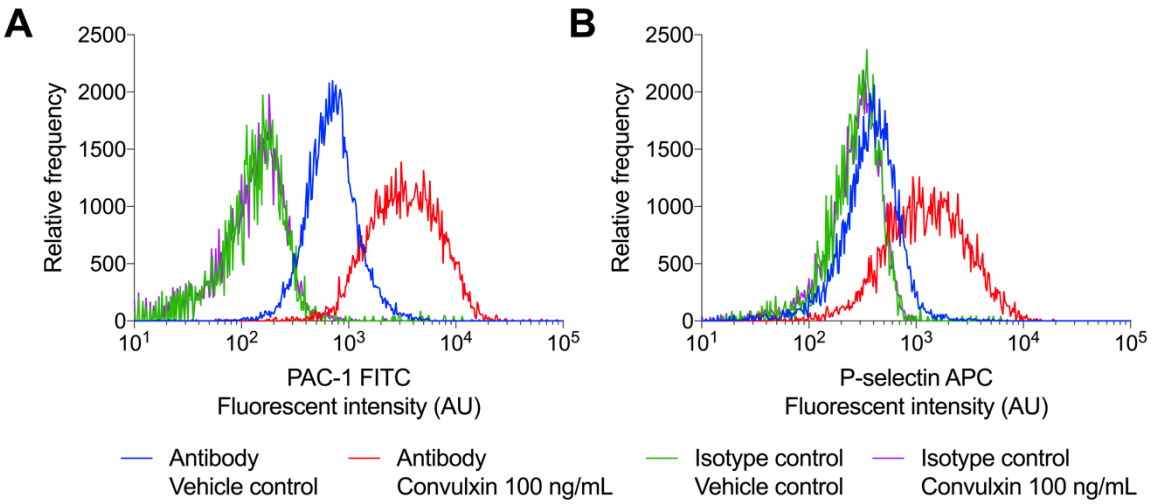


Figure 2-23 Comparing antibody binding with isotype control in droplets (on chip). Platelet suspension pre-incubated with antibody or isotype control and then stimulated with vehicle control or 100 ng/mL convulxin within droplets. After incubation in droplets the emulsion was broken and antibody binding measured on a flow cytometer. **A)** PAC-1 antibody (vehicle in blue and convulxin in red) or isotype control FITC conjugated (vehicle in green and convulxin in purple). **B)** P-selectin antibody (vehicle in blue and convulxin in red) or isotype control APC conjugated (vehicle in green and convulxin in purple). N=1 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table A-2. RA for isotype control (both vehicle and convulxin stimulated) and antibody, vehicle stimulated are all between 0.9 and 1.5. the RA for antibody, vehicle stimulated and isotype, convulxin stimulated compared to antibody, convulxin stimulated were both >10.

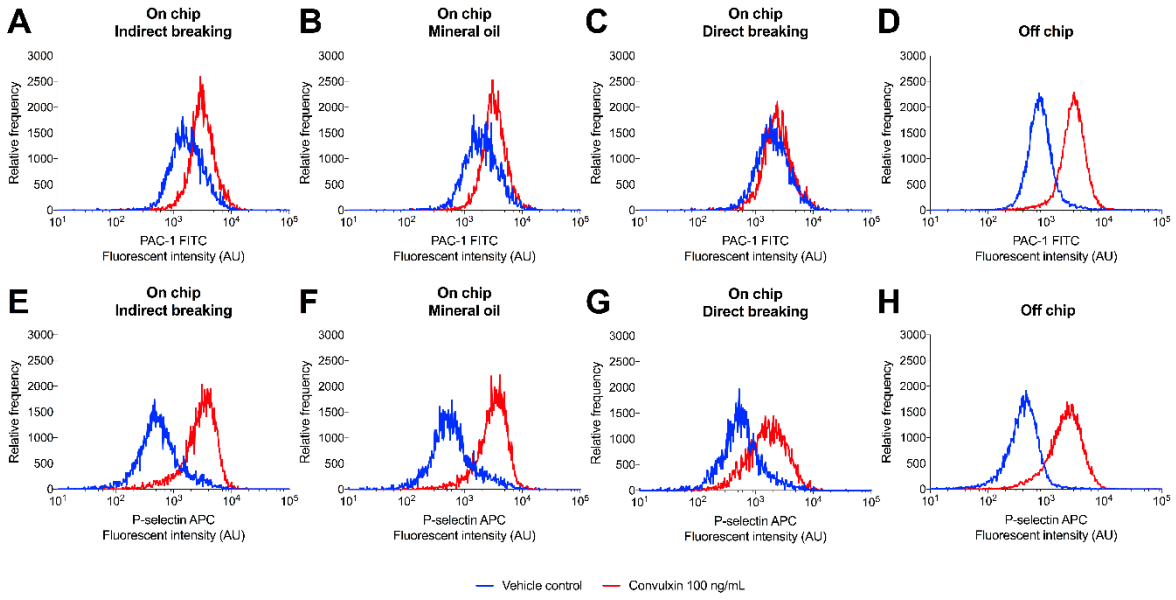


Figure 2-24 The effects of different breaking methods on the signal to noise of measured platelet activity. Platelet activity measured with **A-D)** PAC-1 antibody and **E-H)** P-selectin antibody, comparing stimulation with vehicle (blue) and 100 ng/mL convulxin (red). Different breaking methods were: **A** and **E)** indirect (droplets collected and incubated in a tube before adding fixative and PFO), **B** and **F)** indirect with mineral oil (droplets collected underneath a layer of mineral oil and incubated in a tube before adding fixative and PFO) or **C** and **G)** direct (droplets incubated in long tubing and collected directly in tube with fixative and PFO). Compared with **D** and **H)** off chip control (same platelet suspension with antibodies added to agonist in a tube on the bench before fixation). N=1 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table A-3. RA of vehicle compared to convulxin for indirect breaking and mineral oil were both >5, direct breaking >2 and off chip >10.

2.3.12 Optimization of incubation time improves signal to noise ratio

One way to optimize the signal to noise ratio could potentially be to optimize the incubation time of platelets within the agonist and antibody mixture. To find the optimal incubation time, several incubation times were tested using the pre-incubated platelet suspension (off chip) up to 30 min (Figure 2-25, Figure 2-26 and Table A-4). The measured signal for PAC-1 active platelets increases with time until 20 min (Figure 2-25 and Figure A-15). However, the signal of the PAC-1 antibody in non-stimulated platelets (vehicle control) increases from 20 min onwards. For the P-selectin signal of stimulated platelets an increase can be observed up to 10 min and a slight increase in signal of non-stimulated platelets from 20 min onwards (Figure 2-26 and Figure A-16). An incubation time between 10 and 15 min has the highest signal to noise ratio for both PAC-1 and P-selectin. Therefore, for future experiments droplets were collected during 5 min and incubated on the bench for another 10 min so that all platelets were incubated between 10 and 15 min.

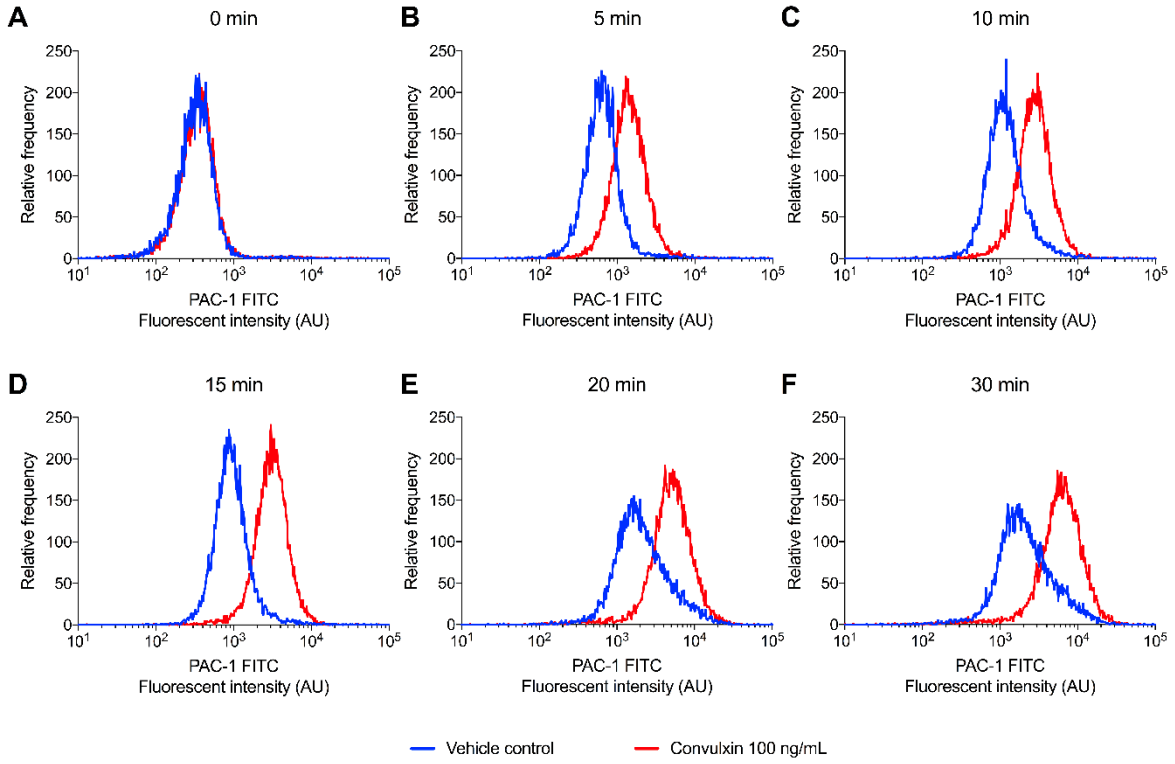


Figure 2-25 Effect of incubation time of a pre-incubated with antibody platelet suspension in an agonist solution on signal to noise of stimulated and non-stimulated platelets. Platelet activity was measured with PAC-1 antibody and platelets were activated with 100 ng/mL of convulxin (red) compared to the vehicle control (blue). Platelets were incubated for **A)** 0 min, **B)** 5 min, **C)** 10 min, **D)** 15 min, **E)** 20 min and **F)** 30 min before addition of fixative to stop the reaction. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table A-4. RA for vehicle compared to convulxin peaks at 15/17.5 min at >20.

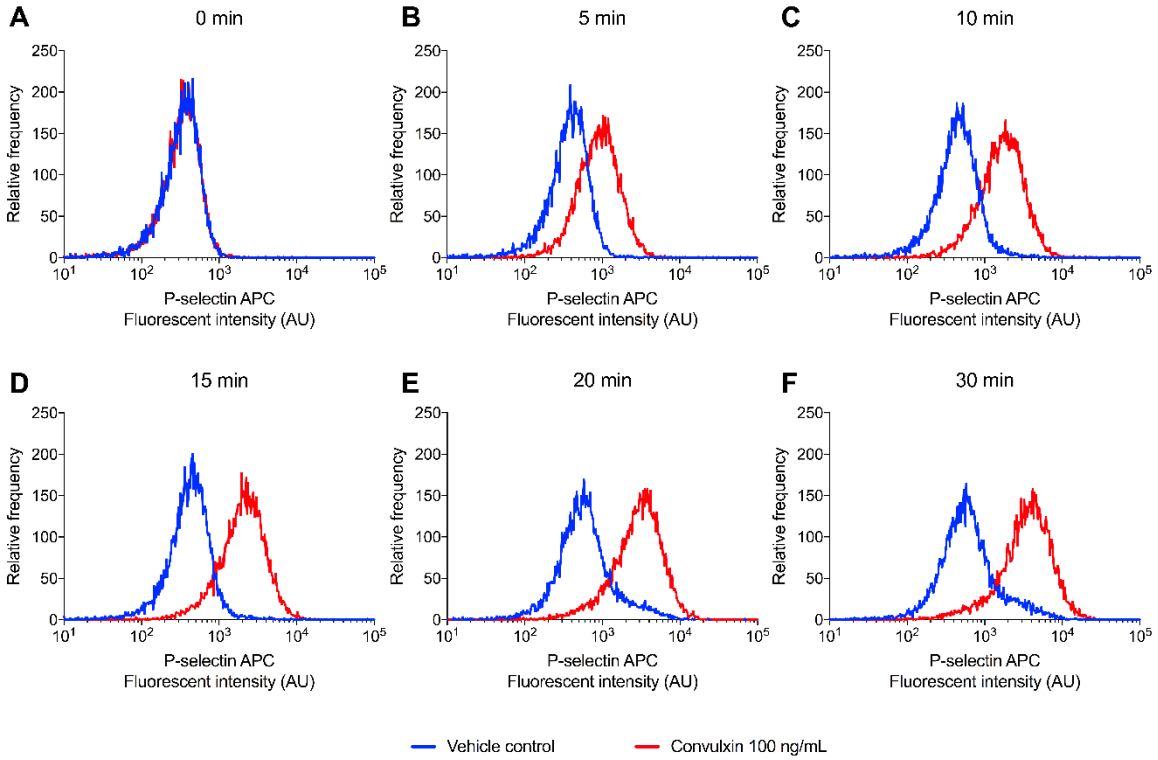


Figure 2-26 Effect of incubation time of a pre-incubated with antibody platelet suspension in an agonist solution on signal to noise of stimulated and non-stimulated platelets. Platelet activity was measured with P-selectin antibody and platelets were activated with 100 ng/mL of convulxin (red) compared to the vehicle control (blue). Platelets were incubated for **A)** 0 min, **B)** 5 min, **C)** 10 min, **D)** 15 min, **E)** 20 min and **F)** 30 min before addition of fixative to stop the reaction. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table A-4. RA for vehicle compared to convulxin continuously rises but reaches >5 at 15 min.

2.3.13 Adding agonist and antibody separately in a 4-inlet device improves signal to noise ratio

The pre-incubation of platelets with antibodies is necessary for cost-effective (as you would lose a lot of antibodies in the dead volume of the syringes with different agonist concentrations) and reliable droplet based platelet assay using a 3 inlet device. However, standard flow cytometry protocols where platelets are added to a mixture of antibody and agonist have a higher signal to noise ratio (Figure 2-15). The adaptation of such a standard protocol in a droplet assay without having to mix the antibody and agonist in a syringe directly, which leads to significant loss of materials during an assay that requires multiple conditions to be tested, requires a 4 inlet device. Such a 4 inlet device needs 2 inlets and a mixing channel before the flow focusing junction that forms it into a droplet with the platelet suspension (Figure 2-2). The signal to noise of this 4 inlet situation greatly improves the signal to noise of mainly PAC-1 (Figure 2-27 and Table A-5). The main mechanism seems to be a reduction in the signal observed with a vehicle control stimulation of platelets. This could possibly be because of the decreased incubation time of platelets with the antibodies (both agonist and antibody are incubated for 10-15 min in the 4 inlet situation but only the agonist incubation is regulated in the 3 inlet situation) which can increase the non-specific binding (Figure 2-25). Another possible explanation could be that something in the antibody solution has a weak activating influence on platelet activity. For future experiments a 4 inlet device was used.

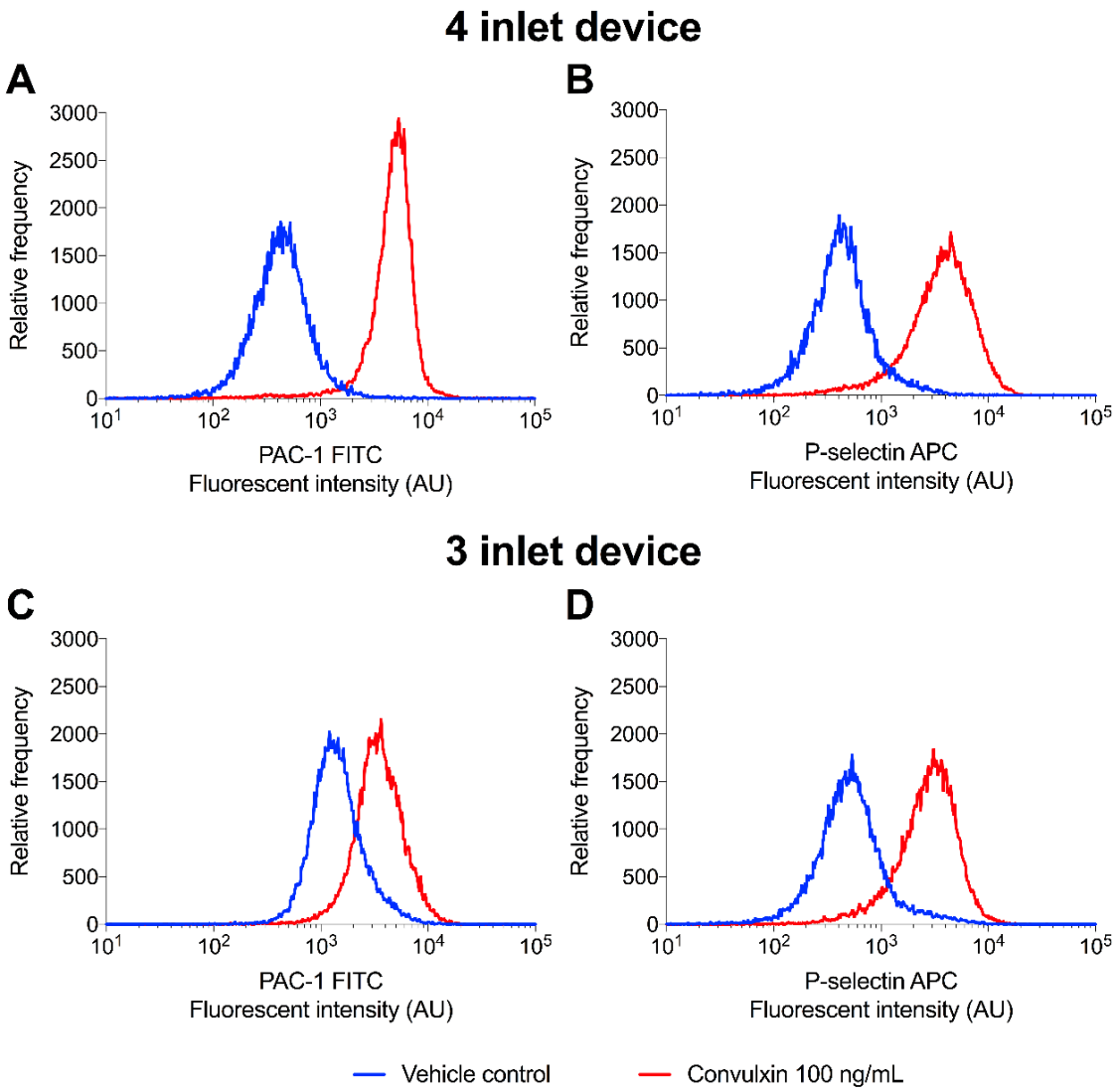


Figure 2-27 Effect of different staining strategies of platelets in droplets. **A** and **B)** platelets were added to an agonist (100 ng/mL convulxin, red or vehicle control, blue) and antibody mixture in the droplets. **C** and **D)** platelets were pre-incubated with antibodies and added to an agonist in the droplets. Platelet activity was measured with **A** and **C)** PAC-1 antibody or **B** and **D)** P-selectin antibody. N=1 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table A-5. RA for vehicle compared to convulxin in the 4 inlet device was 39 and 14 for PAC-1 and P-selectin respectively, while in the 3 inlet device the RA was 13 and 10 respectively.

2.3.14 Single platelet function is reproducible within donors

Variation in assays is a common problem. While inter-donor variation is not a problem in this assay because of the focus on the differences between single and collective platelet function within an individual, intra-donor variation is a potential problem. Also, because single platelet function has not been studied before it is unknown if it is variable over time. To test this, the same donor was measured three times over the course of 9 months with the same method (Figure 2-28, Figure 2-29, Table A-6 and Table A-7). The single platelet function is stable over all three measurements for both PAC-1 and anti-P-selectin. A small decrease in PAC-1 signal on the 3rd repetition can be observed for the collective response. However, the variation is minor for a biological assay and patterns of activation are well preserved.

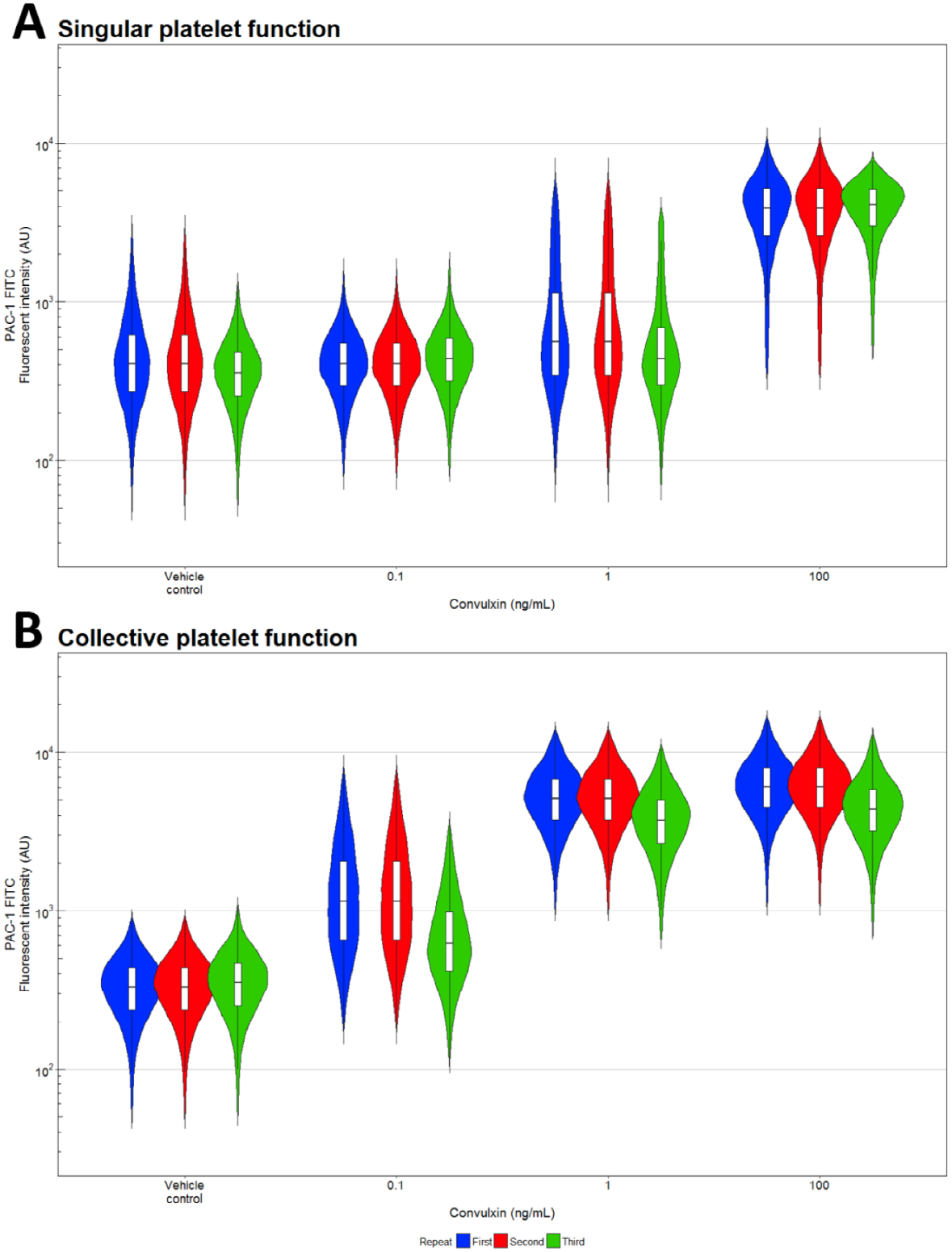


Figure 2-28 Reproducibility of platelet response to convulxin in the same donor. Platelets were stimulated with convulxin **A)** in droplets (singular) or **B)** in suspension (collective). Platelet response was measured with PAC-1 antibody. Three samples were measured taken over a period of 9 months. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile, tails are not trimmed. Repeated measures were done only for one donor (donor 1). Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table A-6 and Table A-7. All single platelet RA were between 0.8 and 2 with one exception (second vs third repeat, convulxin 1 ng/mL) and all collective platelet RA were between 0.5 and 2 with one exception (first vs third repeat, convulxin 1 ng/mL).

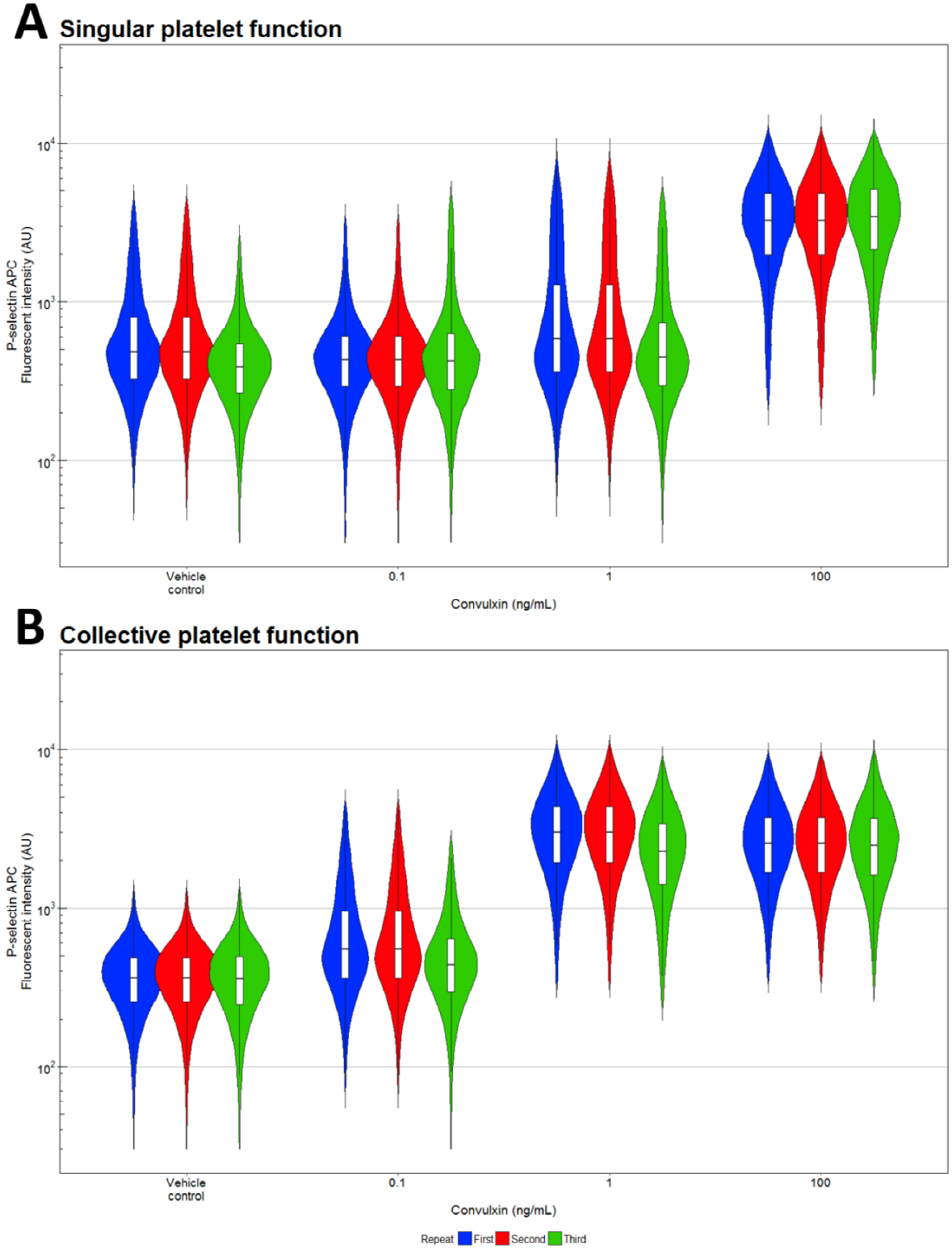


Figure 2-29 Reproducibility of platelet response to convulxin in the same donor. Platelets were stimulated with convulxin **A)** in droplets (singular) or **B)** in suspension (collective). Platelet response was measured with P-selectin antibody. Three samples were measured taken over a period of 9 months. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile, tails are not trimmed. Repeated measures were done only for one donor (donor 1). Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table A-6 and Table A-7. All single platelet RA were between 0.8 and 2 with one exception (second vs third repeat, convulxin 1 ng/mL) and all collective platelet RA were between 0.5 and 2 with one exception (first vs third repeat, convulxin 1 ng/mL).

2.3.15 Single platelet measurements are stable over time

Standard recommendation for platelet assays is to finish all activation measurements within 4 hours after blood draw.[154] However, the assay developed in this study requires different conditions to be measured sequentially rather than simultaneously and has a substantial time required from vein to device. Therefore, the stability of the single platelet measurement over time was tested. First a vehicle control condition was measured and subsequently activated platelets with convulxin (100 ng/mL) were collected every 15 min (Figure 2-30). The vehicle control response over time was not measured because of long switching time between samples (20-30 min) which would make time control impossible. This shows that both single and collective platelet function was stable and reproducible until 6 hours after blood draw but showed a slight decrease thereafter. Therefore, all future experiments were finished before 6 hours after blood draw.

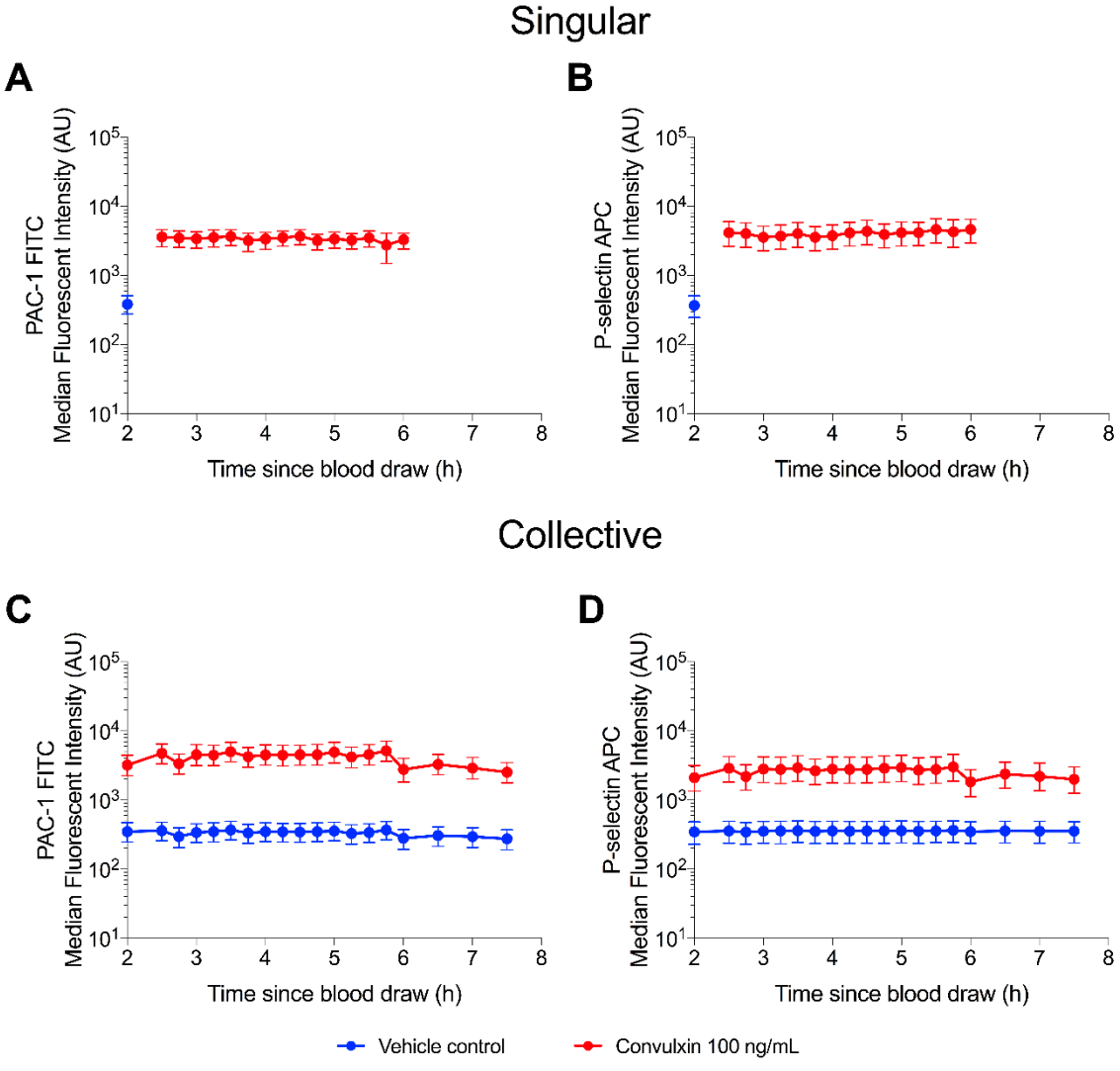


Figure 2-30 Effect of time after blood draw on measured platelet activity **A)** in droplets (singular) measured with PAC-1 and **B)** anti-P-selectin, and **C)** in suspension (collective) measured with PAC-1 and **D)** anti-P-selectin. Platelets are stimulated with either 100 ng/mL convulxin (red) or vehicle control (blue). Singular response was measured by encapsulation within droplets using a 4 inlet device. The gating discussed in section 2.3.5 is applied, and median intensity plotted with quartiles. A minimum of 10000 events within the platelet gate was measured for each condition. Number of independent experiments is 1. Statistical analysis comprised of non-parametric univariate analysis of single platelets.

2.3.16 Single cell investigation in droplets cannot be mimicked by dilution

While the droplet assay developed in this study is a good method of studying single platelets without influences of paracrine signalling, it has been suggested that a simple dilution could give similar results.[33] Because increasing the distance between platelets limits paracrine signalling and most paracrine signals are unstable. To test the effects of platelet dilutions on the collective responses the normal dilution was applied ($5x10^9$/L final concentration) and both 10 and 100 times lower (0.5 and $0.05x10^9$/L) while concentrations of agonist and antibody were kept constant (Figure 2-31). Dilution did not achieve an activation pattern similar to single platelet response in droplets. However, dilution to $0.05x10^9$/L disrupted normal activation and no clear dose response relationship was observed. Also, the effect of dilution in HEPES buffer was compared to dilution in autologous platelet poor plasma (PPP), at $5x10^9$/L only. While the P-selectin response of both diluted in buffer and PPP gained similar responses the PPP dilution decreased the activation observed with PAC-1. Therefore, it can be concluded that encapsulation in droplets in necessary for studying truly single platelet function and purely intrinsic variation, and that dilution in HEPES buffer is used rather than autologous PPP.

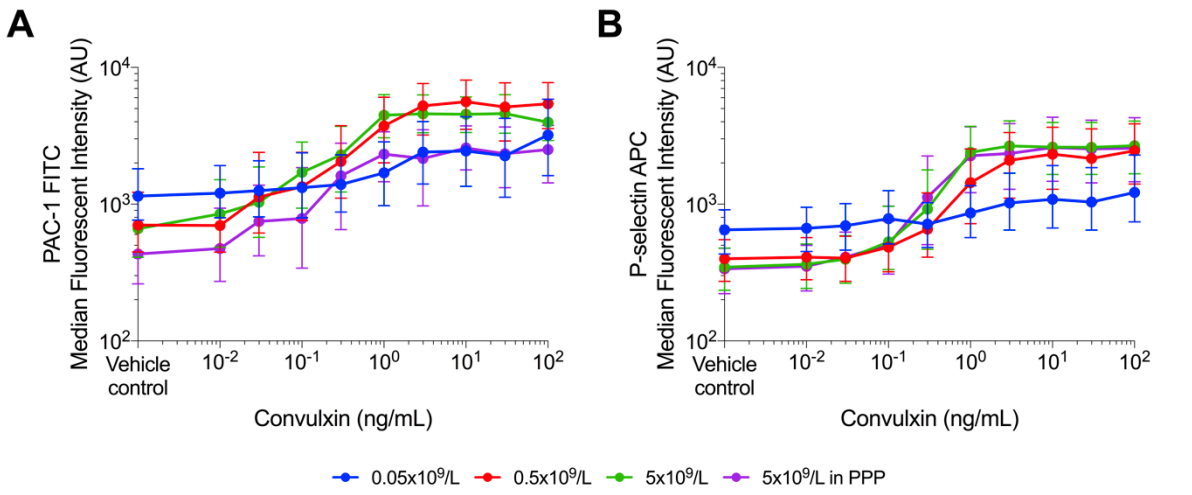


Figure 2-31 The effect of dilutions of the platelet suspension on the response to convulxin stimulation measured with **A)** PAC-1 antibody and **B)** P-selectin antibody. Dilutions used were the standard dilution used in the single cell assay ($5x10^9$/L at junction) with both buffer (green) and autologous platelet poor plasma (purple) and both 10 (blue) and 100 times (red) further diluted with buffer. The gating discussed in section 2.3.5 is applied, and median intensity plotted with quartiles. A minimum of 10000 events within the platelet gate was measured for each condition. Number of independent experiments is 1. Statistical analysis comprised of non-parametric univariate analysis of single platelets.

2.4 Discussion

Platelet variety and heterogeneity, mainly functional variety, has been an increasing interest for the last few decades. However, a method to study intrinsic variety was not available. Without knowledge on functional, intrinsic variety and heterogeneity, thrombus formation cannot be understood, neither in health nor disease. This study has developed an assay combining droplet microfluidics and flow cytometry to create a reliable, high throughput method of studying single platelet function.

This assay is novel in its entirety, not in its individual components per se. The combination of components and adaptation to a new, previously unstudied with droplet microfluidics, cell type. The encapsulation of platelets with antibodies and agonist in droplets, incubation

within droplets, fixation and retrieval, and finally flow cytometric analysis enables the investigation of platelets in total isolation (Figure 2-32).

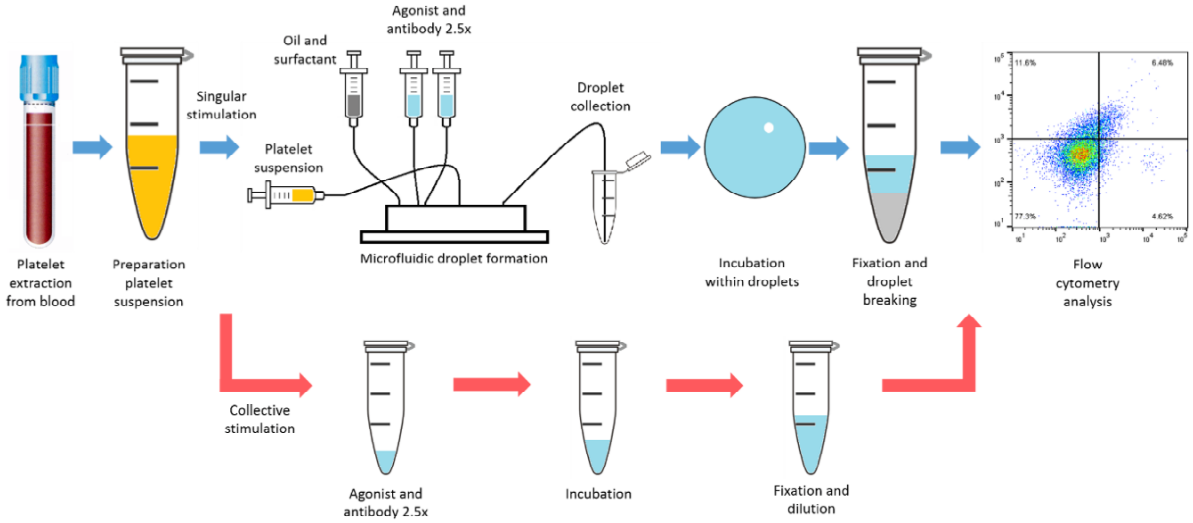


Figure 2-32 Final optimized workflow of single platelet assay. The on chip (single platelets) is above (blue arrows) and the off chip control (collective platelets) below (red arrows).

While this new assay has the capabilities of increasing the understanding of platelet variation and heterogeneity there are some limitations. First, this assay is labour intensive and, compared to flow cytometry only, relatively costly. This extra cost comes from the higher volumes needed to produce a sample, set up time to stabilize the droplets (where surfactant containing oil and platelet reagents are flowing through the device but not producing a sample) and dead volume of syringes. Moreover, the number of conditions measured is limited to around 8-10 maximally, limiting the insights gained per experiment. Additionally, good laboratory practice as is standard in most biological assays, such as triplicate measurements, limits the number of conditions that can be measured further and are left out in this study. Although, the measurement of a high number of single platelets can somewhat compensate this. Another limitation is the dependency of fixing the platelets when breaking the emulsion. As shown before, many antibodies cannot bind their targets on fixed platelets.[78] This limits the use of staining to antibodies and stains that can be included into the droplets (before the fixation) or that are capable of staining fixed platelets and excludes many options. Furthermore, the use of hydrophobic dyes is not an option because of the bulk of oil present in the assay. These can only be used if the fixed platelets are washed and stained after retrieval from the droplets. The current staining protocol involves mixing the agonist and antibody on chip. However, preliminary experiments, performed and kindly shared by Dr. Jonathan West, show that the current serpentine mixing structure is not sufficient for full mixing (Figure A-18). This means that equal amounts of antibody and agonist at junction are dependent on perfectly balanced streams around the platelet inlet. This was monitored throughout with imaging and by checking the droplet sizes, however, this is not ideal and makes the assay even more laborious and dependent on experienced operators. Activation within droplets removes paracrine signalling and thus influence from other platelets but has some limitations. The platelets are activated in suspension only and adherence cannot be studied. Additionally, one of the most important platelet agonists in vivo, collagen, is not suitable for encapsulation due to its tendency to form fibrils and its dependency on a low pH environment. Furthermore, most platelet suspension assays (such as LTA) involve stirring and or mixing. However, in the droplets the platelets are immersed in an agonist and antibody mixture but are not

otherwise being moved. The significance of this is unknown but comparing to standard methods might be difficult.

Regardless of these limitations several further improvements can be made to the existing assay. Improving the mixing structure of the agonist and antibody inlets, for example by a chaotic mixing structure[155], can improve the stability and reliability of the droplet formation and make handling easier. Also, as shown before the incubation time is important for the results but the current assay has an incubation time between 10 and 15 min. While this is sufficient precision for the current antibodies and research aims it might be important for other studies to focus on more precise time controlled reactions. To this effect, the breaking of the emulsion needs to be temporal controlled such as by mixing fixative and PFO on chip. Preliminary experiments have shown this is possible but need further optimization and possibly development of a new device (Figure A-19). Furthermore, the incubation time before the breaking should be temporally controlled. Addition of a channel that corresponds to this incubation time is not practical because of the long length required. Adding a small number of droplets to a plug flow based vector can make temporal control easier because plug flow touches the walls and are therefore not susceptible to overtaking effects of droplets. Another improvement to the assay would be to speed up the time in between conditions as this increases the number of conditions that can be measured within the 6 hour time window available after blood draw. It might be possible to achieve this by modern pressure based controls with valves whereby the switching of a syringe (switching to a different conditions) could theoretically be achieved without a pressure drop and subsequently not take as much time to stabilize after the pressure drop. However, it remains to be seen if the pressure based systems can control the pressure drop enough to prevent having to restabilise the flows. Nevertheless, restabilisation can also be sped up by increasing the size of the devices and therefore the droplets. Although this would increase the use of materials (and the costs) and increase the required dilution, leading to possible dilution artefacts. Balancing the pros and cons of increasing the droplet size is therefore important and dependent on the research aims. However, bigger droplets can also be used to encapsulate small platelet collectives to study the behaviour of thrombus formation in small scale. Addition of coagulation cascade activators can be used to study the effects of coagulation on the response of both single and finite collectives of platelets. If the size of the droplets is sufficiently small these can still be used in a flow cytometer. Moreover, improving imaging techniques of (moving) droplets can increase the information available per experiment. While in this study it is used with platelet rich plasma diluted with HEPES buffer it can easily be applied to use washed platelets or otherwise obtained platelet suspension.

This study has developed a working, integrated platform for the study of single platelet function and functional variety. Additionally, it can be adapted to many more applications in the future. Currently, this assay can be used to study platelet sub-populations and heterogeneity. Increasing our understanding of platelet behaviour on a single platelet level and how this influences their collective responses.

Chapter 3 Hypersensitive platelet subpopulations are demonstrated via single platelet response

3.1 Introduction

Platelets have long since been known to have heterogeneous characteristics, such as size, density, volume and receptor expression levels.[36-38, 59] A wide receptor expression distribution (10-fold between low and high expressing platelets) has been shown for receptors like GPIIIa (CD61), GPVI and GPIbα (CD42b).[59] Whilst maximum expression after activation of P-selectin (alpha granule secretion) has been shown to correlate with $\alpha_{IIb}\beta_3$ integrin activation, CD63 (LAMP-3, dense granules and lysosomes) expression does not correlate with P-selectin expression and it is more scarcely expressed. Other functional heterogeneity that has been widely studied is the procoagulant phenotype (see chapter 4).

Multiple aspects of platelets have been named as likely reasons of this heterogeneity. The origin of platelets from megakaryocytes in the bone marrow has been identified as a possible source of heterogeneity.[40] Two likely mechanisms have been proposed.[59] First, that the heterogeneity of megakaryocytes themselves gives rise to heterogeneous platelets.[156] The second possible mechanism is that the production mechanism, whereby thousands of platelets are produced from a single megakaryocyte, causes heterogeneity in the produced platelets.[9, 10] In addition, the heterogeneity could be caused by the age in circulation. Platelets have a short life span (7-10 days) and limited protein repair machinery. Furthermore, increasing age has historically been associated with a decrease in size, although recently some exceptions have been found, and a decreased size is likely to cause a decrease in response.[33, 48, 59, 157]

The clinical relevance of these heterogeneity findings remains unclear although there is some evidence that an increased percentage of procoagulant platelets increases the risk of coronary heart disease.[158] Single platelet or intrinsic variability studies described in literature usually measure individual platelets within a thrombus by microscopy or in suspension by flow cytometry.[33, 159, 160] However, these methods activate platelets in bulk and are biased by paracrine signalling. Studying single platelet function and intrinsic functional variability requires the exclusion of paracrine signalling, for example by encapsulating in droplets. Therefore, this study aims to:

- Study single platelet function
- Investigate the variety of platelets
- Determine the effect of variety on the collective response of a group of platelets

3.2 Methods

3.2.1 Monoclonal antibodies and reagents

Fluorescent staining was achieved with fluorescein isothiocyanate (FITC) conjugated PAC-1 (PAC-1 clone), allophycocyanin (APC) conjugated CD62P (P-selectin) (AK-4 clone), FITC conjugated anti-CD63 (H5C6 clone) and R-phycoerythrin (PE) conjugated CD42b (HIP1 clone) obtained from Becton Dickinson Pharmingen (San Jose, California, United States). Platelets were activated with convulxin, a snake venom toxin specifically activating the GPVI receptor (main collagen receptor), obtained from Enzo Life Sciences (Exeter, United Kingdom), TRAP-14 from Bachem AG (Bubendorf, Switzerland) and ADP from Sigma Aldrich (Sigma Aldrich, St. Louis, Missouri, United States). Blood samples were diluted in HEPES buffer containing 136 mM NaCl, 2.7 mM KCl, 10 mM HEPES and 2 mM $MgCl_2$ (pH 7.45), stored at 4-7 °C, with glucose 0.1% w/v and BSA 1% w/v added freshly before use.

3.2.2 Participants

Blood was obtained by venepuncture from healthy volunteers after obtaining written informed consent. Ethics approval was obtained from the NRES Committee South Central – Hampshire B (REC reference 14/SC/0211) and from ERGO (protocol number 5538). All participants were free from anti-platelet medication, such as aspirin for 2 weeks and 24 hours free from other non-steroidal anti-inflammatory drugs. Volunteers were evenly distributed among genders (5 males and 3 females) and ages (3 between 20 and 30, 2 each for 30-40 and 40-50 and 1 between 50 and 60). One volunteer was a smoker. The blood was collected using a 21 G needle, into Vacuette tubes containing 1:10 v/v 3.2% trisodium citrate (0.109 M) and gently inverted three times. The first 4 mL was collected into a K_2EDTA containing Vacutainer® tube (BD, Franklin Lakes, New Jersey, United States) and only used for platelet counts.

3.2.3 Blood preparation

Directly after blood collection, citrate tubes were centrifuged at 240 g for 15 min without a brake to prepare platelet rich plasma (PRP). The PRP was set aside for approximately half an hour before use. In the meantime, the platelet count was determined (see section 3.2.4). Subsequently, platelet counts in PRP were adjusted to $100x10^6$/mL and the platelet count measured using the same protocol. Finally, the platelet count adjusted PRP was diluted 1:4 in buffer (final concentration $25x10^6$/mL) and loaded in the syringe or used for controls.

3.2.4 Platelet counting

The platelet count in the PRP and EDTA anti-coagulated whole blood was determined using a protocol by Masters and Harrison, 2014.[147] Briefly, 20 µL of a 20-fold diluted PRP (in HEPES buffer) was stained for 15 min with 1 µL anti-CD61. After the incubation time, this stained sample was further diluted 50-fold. An Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, United States) was used to analyse 100 µL of this diluted sample and platelet counts were calculated from the number of positive events.

3.2.5 Droplet generation

Droplets were generated using a PDMS droplet formation device with inlets for the oil-surfactant mixture, agonist, antibody and platelet solution (Figure 3-1). For droplet generation, fluorinated, gas-permeable oil and surfactant were used, especially designed for live cell assays, 0.75% v/v 008-Fluorosurfactant (Ran biotechnologies, Beverly, Massachusetts, United States) in HFE - 7500 (3M™ Novec™ 7500 Engineered Fluid). Fine bore polythene, medical grade and sterile, tubing with an outer diameter of 1.09 mm and an inner diameter of 0.38 mm (Smiths Medical International, Hythe, United Kingdom) was plugged into the inlets (no separate bonding procedures were used). On the other end, the tubing was pulled over a 25 G needle and connected to a syringe. Pulse free syringe pumps (Fusion 200, Chemyx, Stafford, Texas, United States) were used to infuse the solutions into the devices. Solutions were infused into the devices by pulse free syringe pumps, at speeds of 1200 µL/hr oil, 120 µL/hr agonist, 120 µL/hr antibody and 60 µL/hr diluted platelet solution, to a total flow rate of 1500 µL/hr (flow ratio of aqueous to oil of 1:4, agonist to antibody of 1:1 and platelets to agonist/antibody of 1:4). Live droplet formation was monitored and recorded using a high speed (2500 frames/sec) camera (Vision Research, Wayne, New Jersey, United States) and dedicated software (Phantom Camera Control Software, Vision Research).

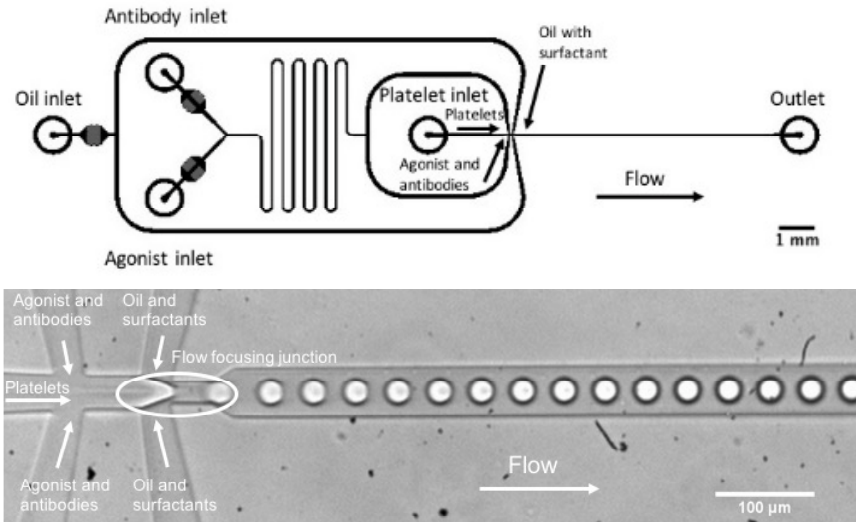


Figure 3-1 Device used to study single platelet function in response to an agonist. The junction where platelets are mixed with the antibody-agonist mixture is 90 µm long and 35 µm wide. This suspension is mixed with oil and surfactant and forced through a pinch section, resulting in droplet formation. Platelets are in the junction for ~0.8 msec and on the chip outside droplets for ~230 msec. After encapsulation the droplets travel along a straight channel for 7 mm (~17 msec) to stabilise the droplet interface.

3.2.6 Flow cytometry

Droplets were collected for 5 min continuously and incubated in the dark for 10 min to ensure all platelets were incubated between 10 and 15 min which was determined to be the optimum incubation time. Afterwards 200 µL fixative and 200 µL 1H,1H,2H,2H-perfluoro-1-octanol (Sigma Aldrich, St. Louis, Missouri, United States) was added to destabilise the droplet interface. After a few minutes the supernatant was taken off and measured immediately on the flow cytometer (Accuri C6 flow cytometer from BD Biosciences).

Controls samples were stimulated in the same ratios as the droplet generation runs (agonist to antibody of 1:1 and platelets to agonist/antibody of 1:4), 10 µL agonist, 10 µL antibody and 5 µL of platelet solution. After 15 min of adding the platelet solution, 100 µL of fixative is added to stop further platelet activation and antibody binding. Samples were incubated in the fridge and measured within 4 days. Before cytometry, 100 µL of PBS was

added to dilute the platelets. Unless otherwise stated the antibodies used were PAC-1 (1.25 ng/µL), anti-CD42b (1.25 ng/µL) and anti-P-selectin. (0.63 ng/µL)

3.2.7 Statistical analysis

Droplet videos were analysed using ImageJ software (open source[161]). Flow cytometry samples were analysed with FLOWJO (Ashland, Oregon, United States). Graphs and statistical analysis were performed using GraphPad Software (Prism) (San Diego, California, United States) or R (open source). Significance testing of single platelet experiments was performed with the relative risk / risk ratio procedure plus confidence intervals. For these single platelet experiments this test was named relative activation.

Platelets were selected with a gate on the CD42b antibody fluorescence intensity and subsequently doublets were removed with gates on the forward scatter area vs height and forward scatter height vs width. Lastly a gate was placed on the forward scatter height vs sideward scatter height to gate out non-platelet sized events (see Chapter 2). Collective samples were performed in triplicate and subsequently a representative sample was selected. This selection was based on the median of triplicate values and the sample which was most often the median in 7 parameters (FSC-A, FSC-H, SSC-H, FL1-H, FL2-H and FL4-H) was selected. Ties were broken with the median of the FL1-H parameter.

3.3 Results

3.3.1 Paracrine signalling is important for platelet sensitivity

Platelet response to stimuli includes secretion of granule content that enhances its own function (such as extra copies of the $\alpha_{IIb}\beta_3$ receptor) and activates platelets in the vicinity. Because this platelet-platelet amplification interferes with the ability to study single platelet function, this study encapsulates platelets within water-in-oil droplets. Platelet function was compared between platelets within droplets (singular) and platelets in suspension (collective) by stimulating with increasing concentrations of convulxin, a specific GPVI activator. Both single and collective platelet response show a clear dose-response (S) curve shape (Figure 3-2). The singular platelet response is more variable than the collective response, but all blood donors showed a similar pattern. P-selectin expression is less variable among the donors than integrin $\alpha_{IIb}\beta_3$ activation (Figure 3-2). However, both alpha granule secretion and integrin activation show a markedly reduced sensitive singular platelet response to convulxin compared to the collective response.

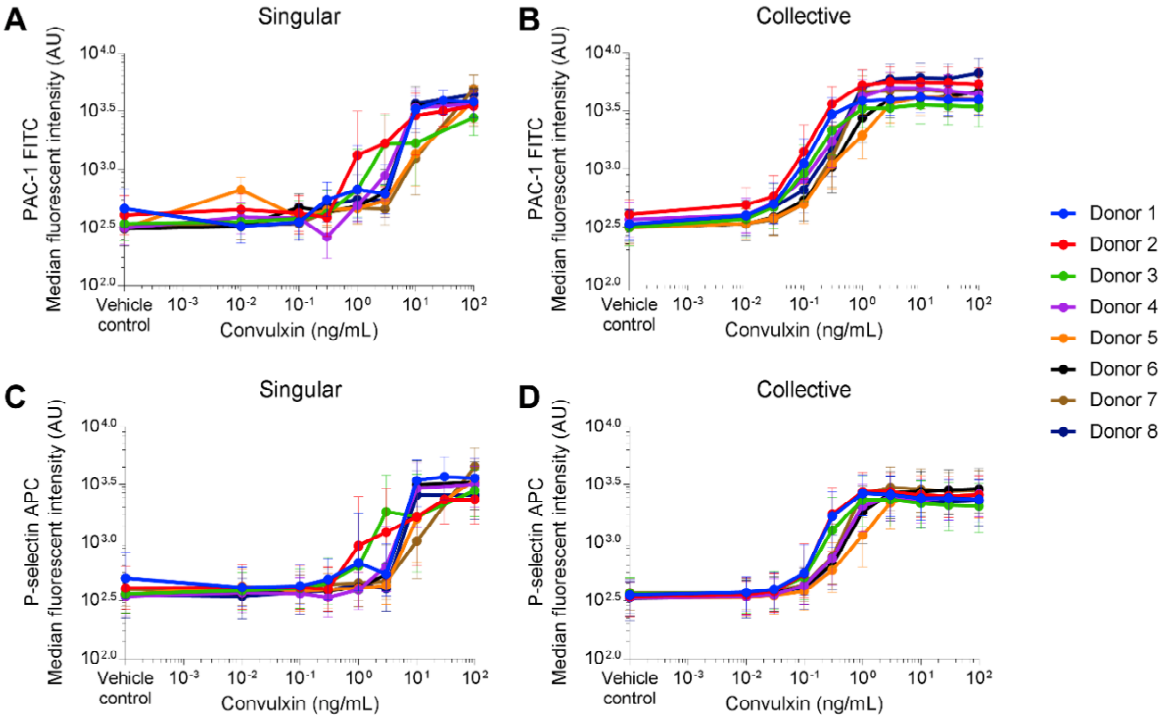


Figure 3-2 Dose-response curves of convulxin and median fluorescent intensity of the **(A-B)** PAC-1 antibody (binding the active $\alpha_{IIb}\beta_3$ receptor) and **(C-D)** P-selectin antibody (indicating alpha granule secretion) binding. Platelets were activated **(A and C)** singularly, within droplets and (**B and D)** collective, in suspension. The point on the Y-axis is the vehicle control. Convulxin is used at concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 ng/mL (singular response was not measured with 0.03 and 30 ng/mL). Results are shown as median with 25th and 75th percentiles. N=8 Statistical analysis comprised of the relative activation (RA) with confidence intervals (see Figure 3-3 and Figure B-1) and an Emax model was fitted (Figure 3-4).

When quantifying the difference between single platelets and collectives the chi-squared test can be useful. However, the chi-squared test does not test direction nor the magnitude of the response which is very important in this case. Therefore, the relative activation was used instead. The resultant relative activation surrounded by the confidence interval was plotted (Figure 3-3). This shows a pattern of a negative effect for the vehicle control (shown by a relative activation lower than 1 and a confidence interval not including 1) and a small positive effect for very low concentrations of convulxin. Adding medium concentrations of convulxin leads to high relative activation culminating in a peak at 3 ng/mL. The relative activation returns to a small positive effect for high concentrations showing a small difference between single and collective platelet response. This pattern is similar for both PAC-1 and P-selectin but the magnitudes are quite different (53 and 8 respectively). The relative activation reaches a maximum for 3 ng/mL convulxin (or 1 ng/mL in 2 donors) and exceeds 20 for PAC-1 and 4 for P-selectin for all donors (Figure B-1 and Table B-2 to Table B-9). Furthermore, when an Emax model (Figure 3-4 and Table B-1) was fitted the EC50 values of singular and collective response were more than 10-fold different (7.5 ng/mL compared to 0.4 ng/mL). So paracrine signalling is important for platelet sensitivity to convulxin because in the absence of paracrine signalling the sensitivity is starkly reduced.

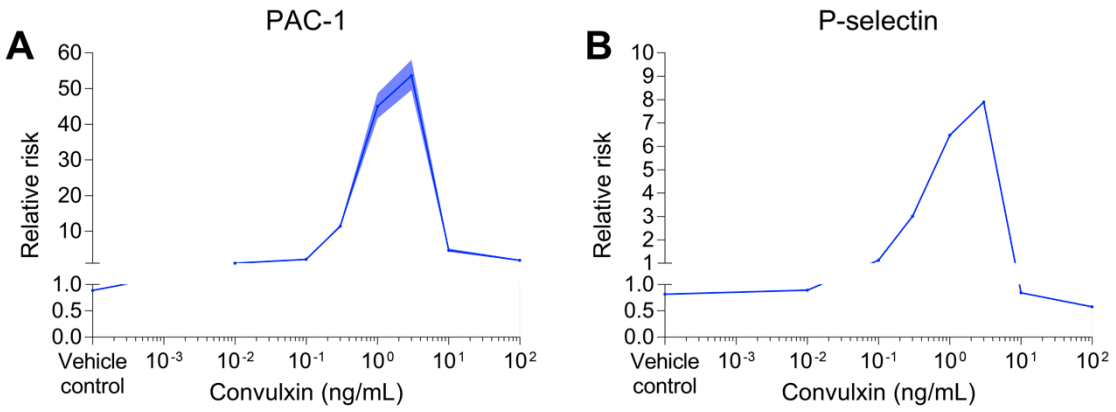


Figure 3-3 The relative activation of a positive platelet response for singularly stimulated compared to collectively stimulated platelets. Platelet response was measured with **A)** PAC-1 (integrin $\alpha_{IIb}\beta_3$ activation) and **B)** P-selectin (alpha granule secretion). Data is shown as relative activation with confidence interval of donor 1. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

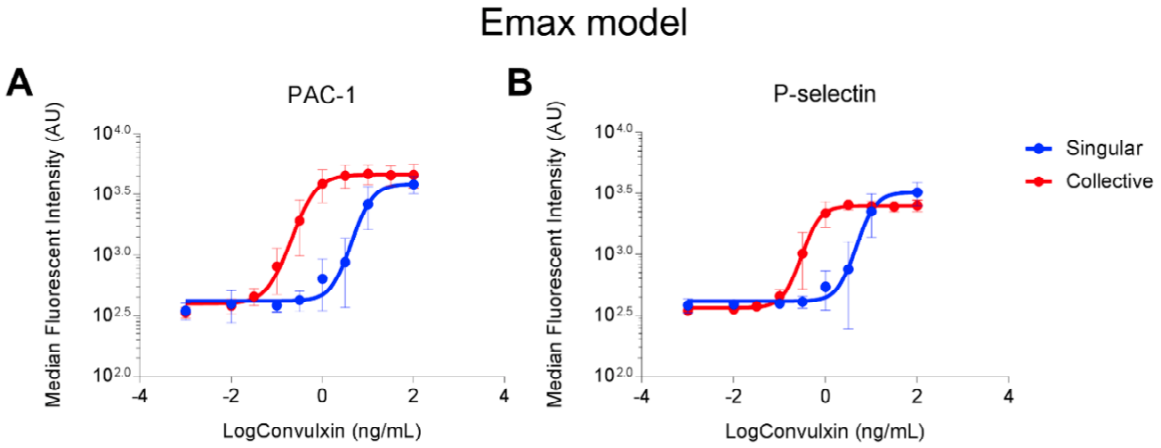


Figure 3-4 The Emax model for the dose-response relationship between convulxin dosing and **A)** PAC-1 or **B)** P-selectin expression. Results are shown as median with quartiles for 8 donors and the Emax model trend line. Details of the fitted model can be found in Table B-1.

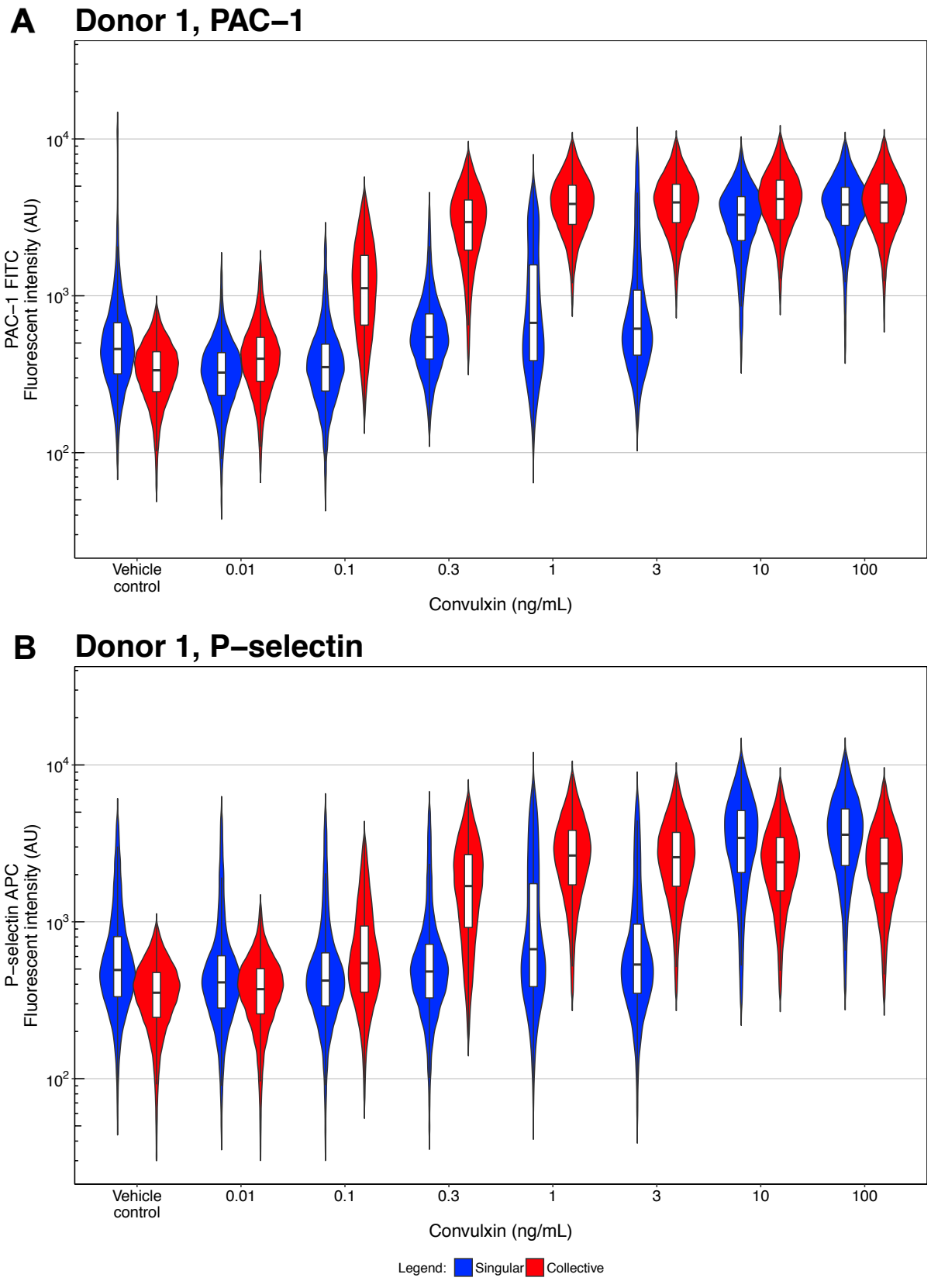


Figure 3-5 Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile, tails are not trimmed. N=8 (also see Figure B-2 to Figure B-8). Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-2. The RA for singular compared to collective for PAC-1 activation peaks at 3 ng/mL convulxin at 53 and for P-selectin expression at 3 ng/mL convulxin at 8. Singular RA vehicle compared to convulxin stimulation peaks at 100 ng/mL at 28 and 12 respectively for PAC1 and P-selectin, while collective RA peaks at 10 ng/mL at 78 for PAC-1 and at 3 ng/mL convulxin at 10 for P-selectin. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

3.3.2 Stimulation with convulxin triggers various sensitivity levels

Stimulating platelets with convulxin in the absence of paracrine signalling reveals variable sensitivity levels (Figure 3-5). The integrin activation of single platelets has a similar maximum and minimum activation pattern as platelet collectives although at different concentrations of convulxin (see section 3.3.1). Single platelet stimulation with higher concentrations of convulxin show a subpopulation of platelets that is hyposensitive which is absent in collectively stimulated platelets. Medium concentrations divide the single platelets into active and non-active platelets rather than transition states (see section 3.3.5). Stimulation with low concentrations reveals a small hypersensitive subpopulation of platelets (see section 3.3.3).

P-selectin expression on the plasma membrane is related to the integrin $\alpha_{IIb}\beta_3$ activation but shows less clearly distinct sensitivity patterns. Hyposensitivity is also present in very high concentrations of convulxin (100 ng/mL) which is also observed in the presence of paracrine signalling. Intermediate activity levels are more binary than collectively stimulated platelets but not as much as observed with PAC-1 binding (see section 3.3.5). However, the hypersensitive population appears similar in distribution to that observed with integrin activation.

3.3.3 Stimulation with low concentration convulxin reveals an intrinsic hypersensitive subpopulation

Stimulation with low concentrations of convulxin leads to a heterogeneous platelet response. Most platelets have a similar fluorescence intensity to negative platelets (unstimulated, vehicle control) but a small subpopulation of platelets has a much higher fluorescent intensity (Figure 3-6). The same concentration of convulxin leads to full (or near-full) activation in collectively stimulated platelets. This hypersensitive population can only be observed in singularly stimulated platelets. These platelets responded by both activation of the integrin $\alpha_{IIb}\beta_3$ and secretion of the alpha granules.

The concentration at which the hypersensitive population becomes visible is variable among individuals. In most individuals the hypersensitive population are observed at a concentration of 1 or 3 ng/mL (Figure B-2 to Figure B-8). The size of the subpopulation of platelets varies between individuals with the maximum size around 25%. Also, the population of hypersensitive platelets is at least 10-fold more sensitive than the bulk of the platelets. For example, the 25% hypersensitive platelets seen in Figure 3-6 are activated at 1 ng/mL while the percentage of active platelets approaches 90%. only at a concentration of 10 ng/mL Furthermore, the first left to right shift in the corresponding collective sample can be observed at 0.1 ng/mL, although the transition pattern is different (see section 3.3.5).

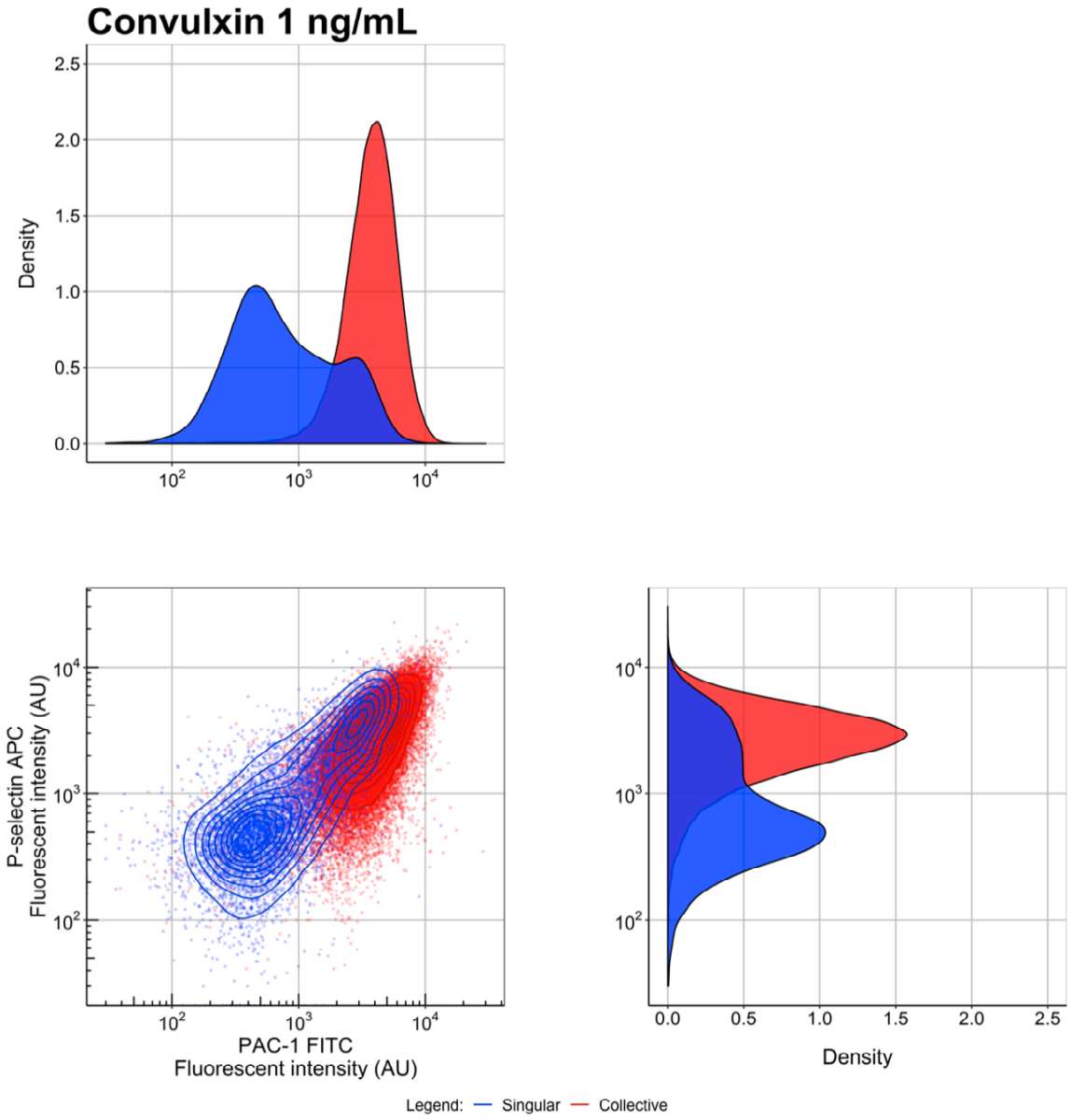


Figure 3-6 Relationship between PAC-1 binding and P-selectin expression of singular (blue) and collective (red) platelets stimulated with 1 ng/mL of convulxin. Data is selected from the experiment depicted in Figure 3-5 (donor 1). Data is shown as scatter plot with contour plot showing densities (bottom left) and corresponding density plots for both PAC-1 (top) and P-selectin (bottom right) only. The hyperactive, singular platelet population 35% of all singular platelets. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

3.3.4 The hypersensitive population has no different forward- and sideward scatter

The hypersensitive subpopulation is more sensitive than the rest of the platelets leading to a response to low concentrations of convulxin. However, it is interesting to study other aspects of these platelets to see if the hypersensitive subpopulation consists of a distinct phenotype of platelets. The hypersensitive subpopulation was selected by plotting all single platelets stimulated with a low concentration of convulxin (1 ng/mL for donor 1) and selecting those that were positive for both PAC-1 and P-selectin (Figure 3-7A). The scatter pattern of these two populations (hypersensitive and the rest of the single platelets) was compared. Forward scatter (an indication of platelet size) and sideward scatter (an indication of platelet granularity) were compared (Figure 3-7B). The forward and sideward scatter of platelets shows a wide variety between individual platelets. However, the

hypersensitive population and the "normal" population overlap and are indistinguishable from each other. When comparing the forward scatter and CD42b (GPIb) fluorescent intensity the populations largely overlap although the hypersensitive population has a slightly lower CD42b signal than the normal population (Figure 3-7C). A slight loss in CD42b signal is associated with platelet activation (Figure B-9).

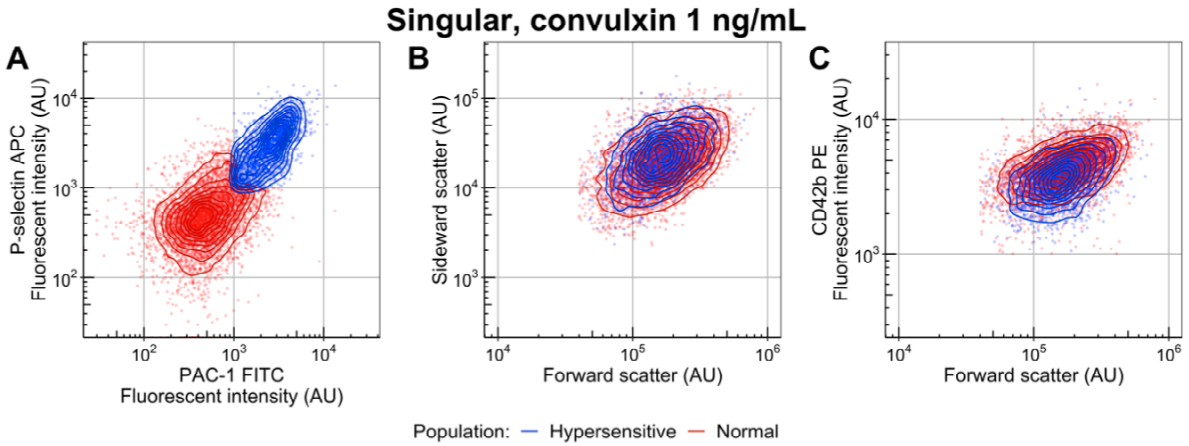


Figure 3-7 Comparing the hypersensitive platelet subpopulation (blue) with the rest of the single platelets (red). Comparing **A)** Activity levels measured by PAC-1 (integrin $\alpha_{IIb}\beta_3$ activation) and P-selectin (alpha granule secretion), **B)** Forward scatter (an indication of size) and sideward scatter (an indication of granularity) and **C)** Forward scatter and CD42b (GPIb) expression. Data is the singular platelet sample shown in Figure 3-6 (single platelets of donor 1 stimulated with 1 ng/mL). A fluorescent intensity >1000 AU for both PAC-1 and P-selectin was used to determine positive platelets (hypersensitive platelets). Data is shown as scatter plots with contour plots showing densities.

3.3.5 Intermediate single platelet response shows discrete populations and no transitional state

When stimulating single platelets with medium concentrations of convulxin, so that approximately half of the platelets are activated, an interesting pattern emerges (Figure 3-8A). It shows two distinct peaks and a trough in the middle, indicating two discrete populations of non-active and active platelets (for PAC-1 binding). Platelets in suspension (collective) that are stimulated with convulxin to cause half of the platelets to activate have a very different pattern (Figure 3-8B). There is only one, very broad, peak where most of the platelets are located in the middle i.e. have intermediate activation level. This transition state is observed in all donors but only in the presence of paracrine signalling. The secretion of the alpha granules follows a similar but less distinct pattern (Figure B-10).

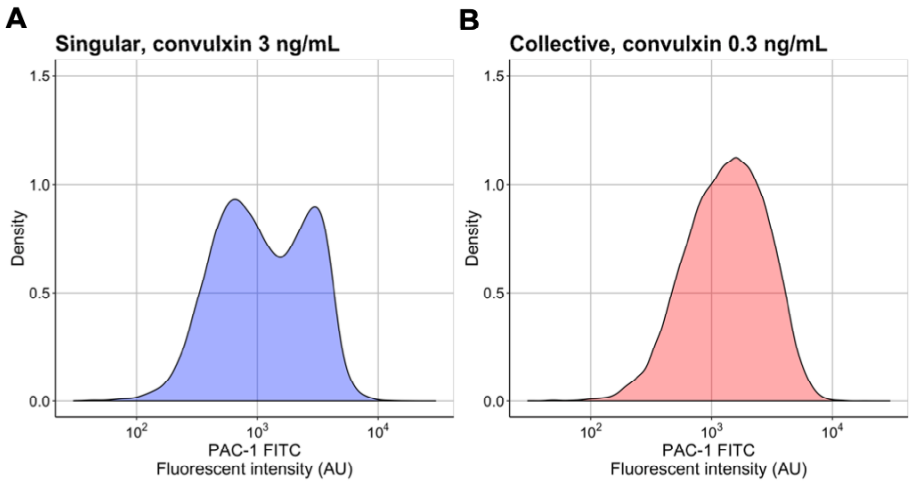


Figure 3-8 Density plots of the intermediate platelet response to convulxin. Intermediate response shown for **A)** single platelets to 3 ng/mL convulxin and **B)** collective platelets to 0.3 ng/mL convulxin. Samples from donor 1 and same day (see Figure 3-6).

Paracrine signalling can be an explanation for the transition state with both the extent of and distance to the signalling platelet determines the level of activation of the other platelet. However, it can also be that the restricted volume of a droplet enhances autocrine signalling. To test this, platelets were encapsulated in droplets of approximately twice the diameter (using a 50 by 50 μm junction), which is equivalent to an ~8-fold larger droplet volume (the concentration of platelets had to be adjusted to ensure single platelet encapsulation), and compared to normal droplets (Figure 3-9). This shows a mixed picture with some platelets in a transition state and some in a, distinctly, active state. The pattern for P-selectin is less clear and is more similar to the collective, intermediate state.

Another reason for this could be the limited number of molecules, of a relatively large and very potent molecule like convulxin (~84 kDa), that are encapsulated in the droplets. In bigger droplets there are more molecules encapsulated at the same concentration than in smaller droplets. To test the effect of the number of molecules available per platelet, the concentration of platelets was adjusted to account for all empty droplets (Equivalent molecules, Figure 3-10 and Figure B-11). This shows an activity pattern between the small droplets and the collectively stimulated platelets but more similar to the collectives than the big droplets.

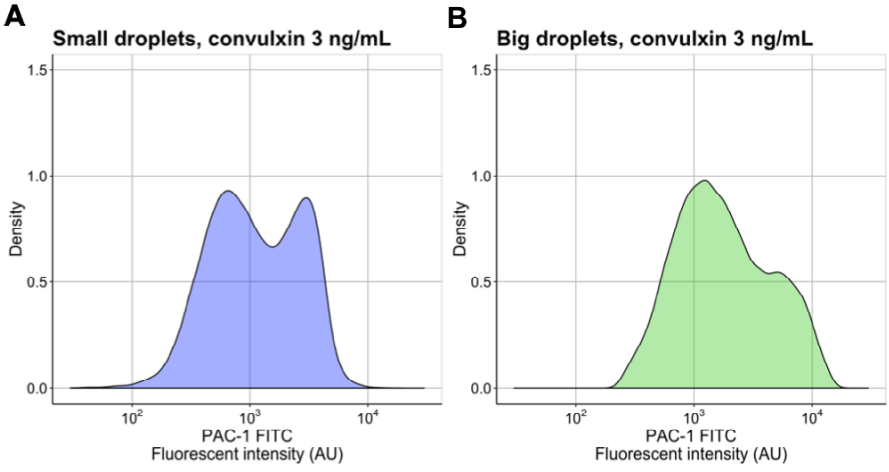


Figure 3-9 Density plots of the intermediate platelet response to convulxin. Intermediate response shown for single platelets in **A)** normal, small, droplets (∅25 μm) and **B)** bigger droplets (∅55 μm) , both to 3 ng/mL convulxin. Samples from donor 1 but measured on different days.

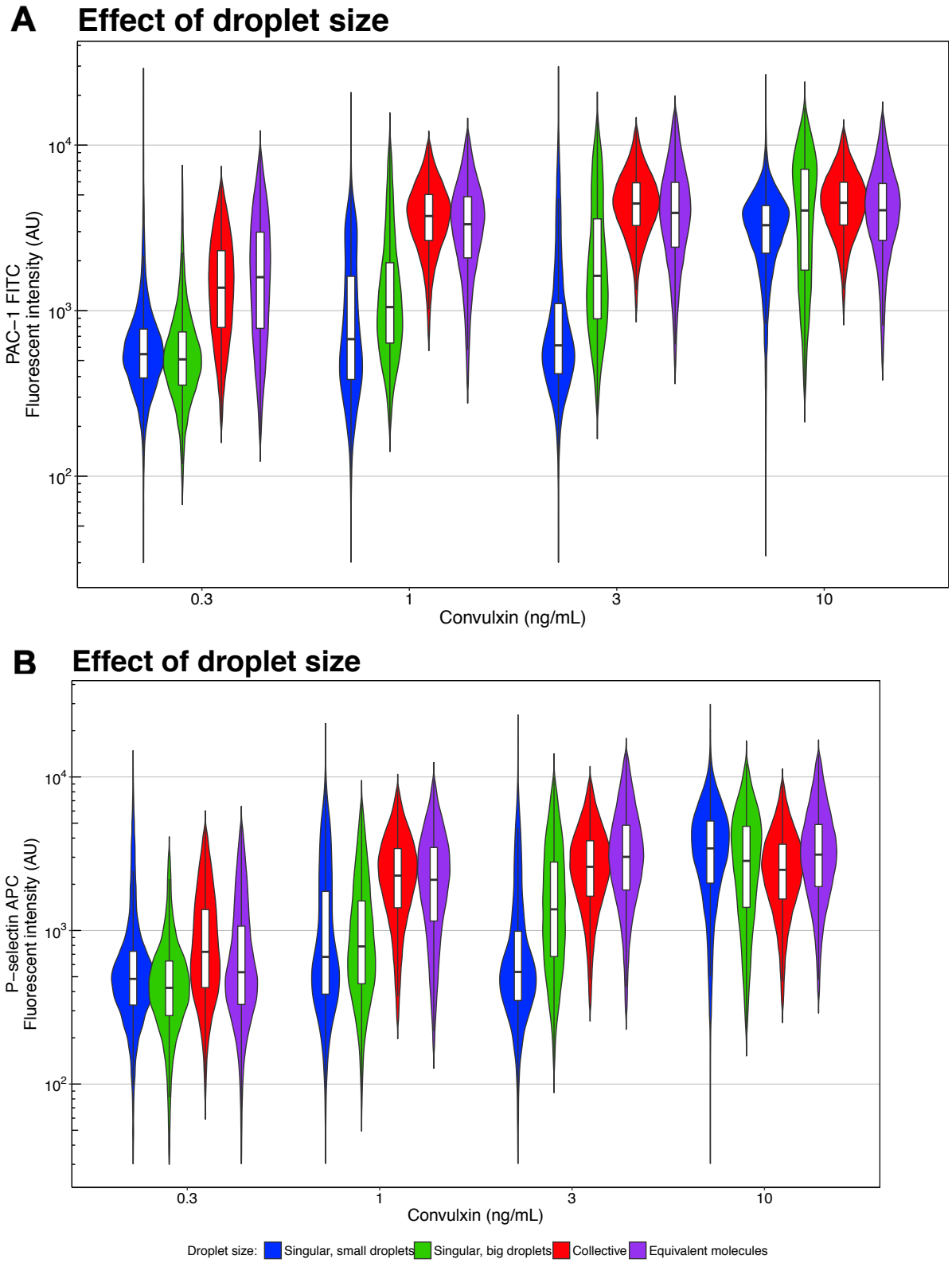


Figure 3-10 Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to convulxin stimulation in standard droplets of ~25 µm diameter (singular, small droplets, blue), in bigger droplets of ~55 µm diameter (singular, big droplets, green), in suspension (collective, red) or in suspension with a 25 times higher concentration of platelets (equivalent molecules, purple), to correct for convulxin molecules found in empty droplets. These were not measured on the same day but samples are obtained from the same donor (donor 1). The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. Singular, small droplets N=12, singular, big droplets N=2, collective N=12, equivalent molecules N=3. Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-10. The RA for singular small droplets compared to big droplets for PAC-1 activation peaks at 10 ng/mL convulxin at 6.7 and for P-selectin expression at 5.1. The RA for collective compared to equivalent molecules for PAC-1 activation has the highest difference at 3 ng/mL convulxin at 0.2 and for P-selectin expression at 3 ng/mL convulxin at 0.7. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

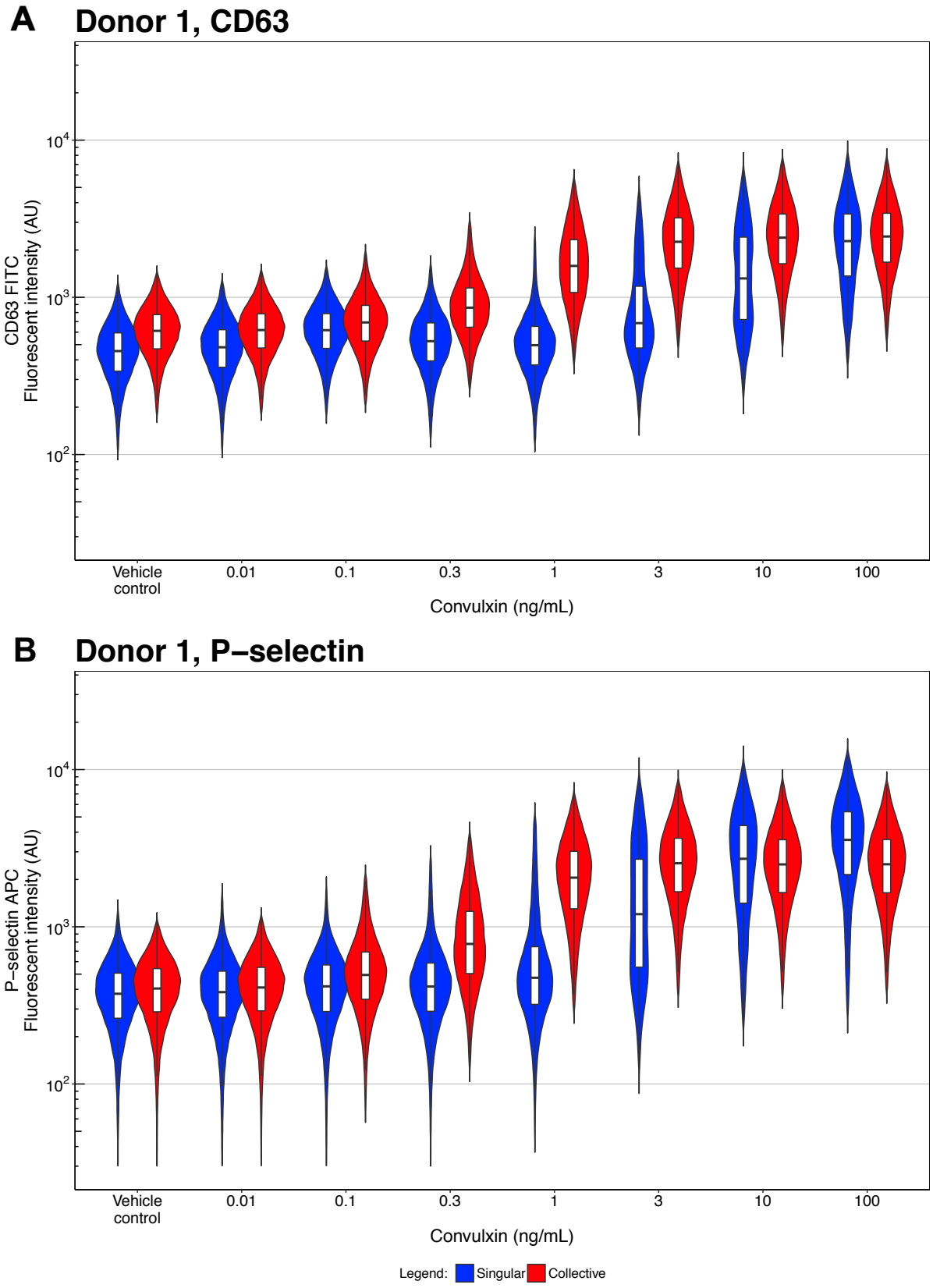


Figure 3-11 Violin and box plot of the fluorescent intensity observed with **A)** CD63 (dense granule secretion) or **B)** P-selectin antibody in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD61 and following the gating procedure described in section 2.3.5. The violin plot is formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. N=2 (also see Figure B-13). Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-11. The RA for singular compared to collective for CD63 activation peaks at 3 ng/mL convulxin at 7.2 and for P-selectin expression at 1 ng/mL convulxin at 5.1. Singular RA vehicle compared to convulxin stimulation peaks at 100 ng/mL at 6.4 and 10 respectively for CD63 and P-selectin, while collective RA peaks at 100 ng/mL at 13 for CD63 and at 10 for P-selectin. A fluorescent intensity >1000 AU was used to determine positive platelets (for both CD63 and P-selectin).

3.3.6 Dense granules are secreted by the hypersensitive subpopulation

The hypersensitive subpopulation discussed in the previous sections was shown to have equal integrin $\alpha_{IIb}\beta_3$ activation and alpha granule secretion. However, the dense granules have not been included in the analysis of the hypersensitive subpopulation. To look at the relationship between alpha granule and dense granule secretion, single platelets were co-stained with anti-CD63 and anti-P-selectin. The dose-response curve looks similar to that of P-selectin but flatter, indicating a lower total response (Figure B-12). Some platelets never become positive to CD63 (Figure 3-11). This is similar to P-selectin although a bigger percentage of platelets are non-responders to convulxin of CD63 than P-selectin. Also similar to P-selectin is the higher concentration of convulxin required for a response in single compared to collective platelet stimulation. In contrast to P-selectin is the concentration of convulxin required for a CD63 response. When comparing both upon stimulation, of both single and collective platelets, with 1 ng/mL of convulxin a small population of P-selectin but CD63 negative platelets is shown (Figure 3-12). At a concentration of 3 ng/mL a distinctive pattern is shown (Figure 3-13). Some platelets are negative for both, some platelets are positive for P-selectin only and some are positive for both P-selectin and CD63. No platelets are positive for CD63 and negative for P-selectin. At a concentration of 10 ng/mL a dual pattern of activation can be observed for single platelets, a pattern never observed for collectively stimulated platelets (Figure 3-11). Similar observations can be made for a second blood donor (Figure B-13).

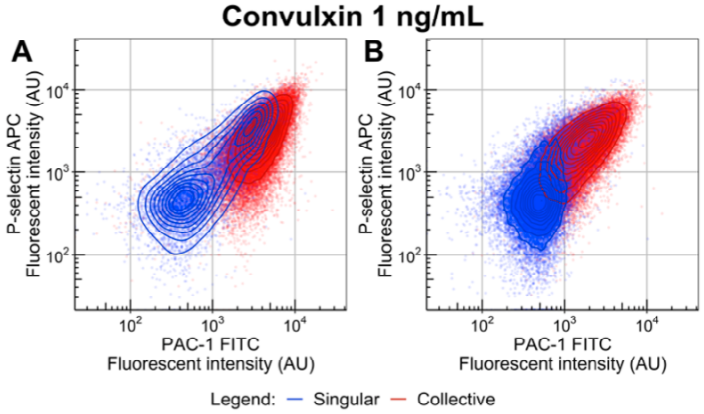


Figure 3-12 Relationship between PAC-1 binding, P-selectin and CD63 expression of singular (blue) and collective (red) platelets stimulated with 1 ng/mL of convulxin. Scatter plot of **A)** PAC-1 and P-selectin expression and **B)** CD63 and P-selectin expression. Data is selected from two different experiments depicted in Figure 3-5 and Figure 3-11. Data is shown as scatter plots with contour plots showing densities.

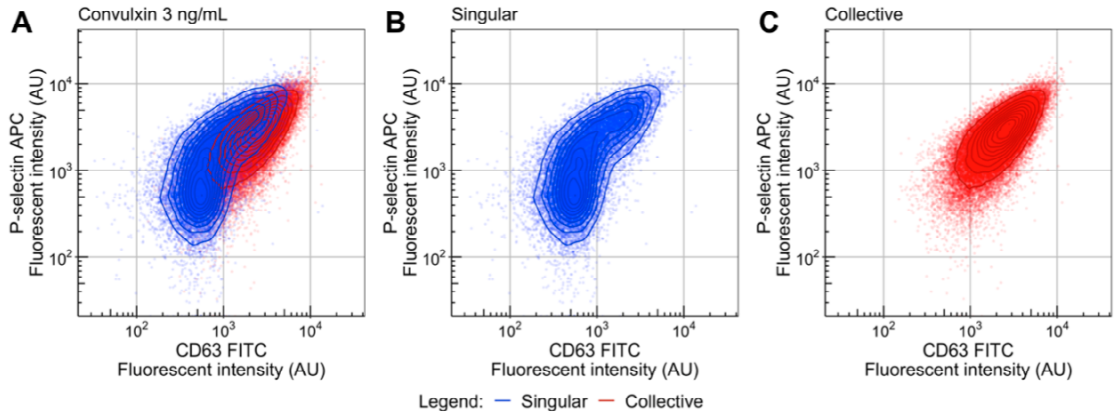


Figure 3-13 Relationship between CD63 (dense granule secretion) and P-selectin (alpha granule secretion) expression of singular (blue) and collective (red) platelets stimulated with 3 ng/mL of convulxin. Scatter plot of **A)** overlay of singular and collective platelet response, **B)** only singular and **C)** only collective platelet response. Data is selected from the experiment depicted in Figure 3-11. Data is shown as scatter plots with contour plots showing densities.

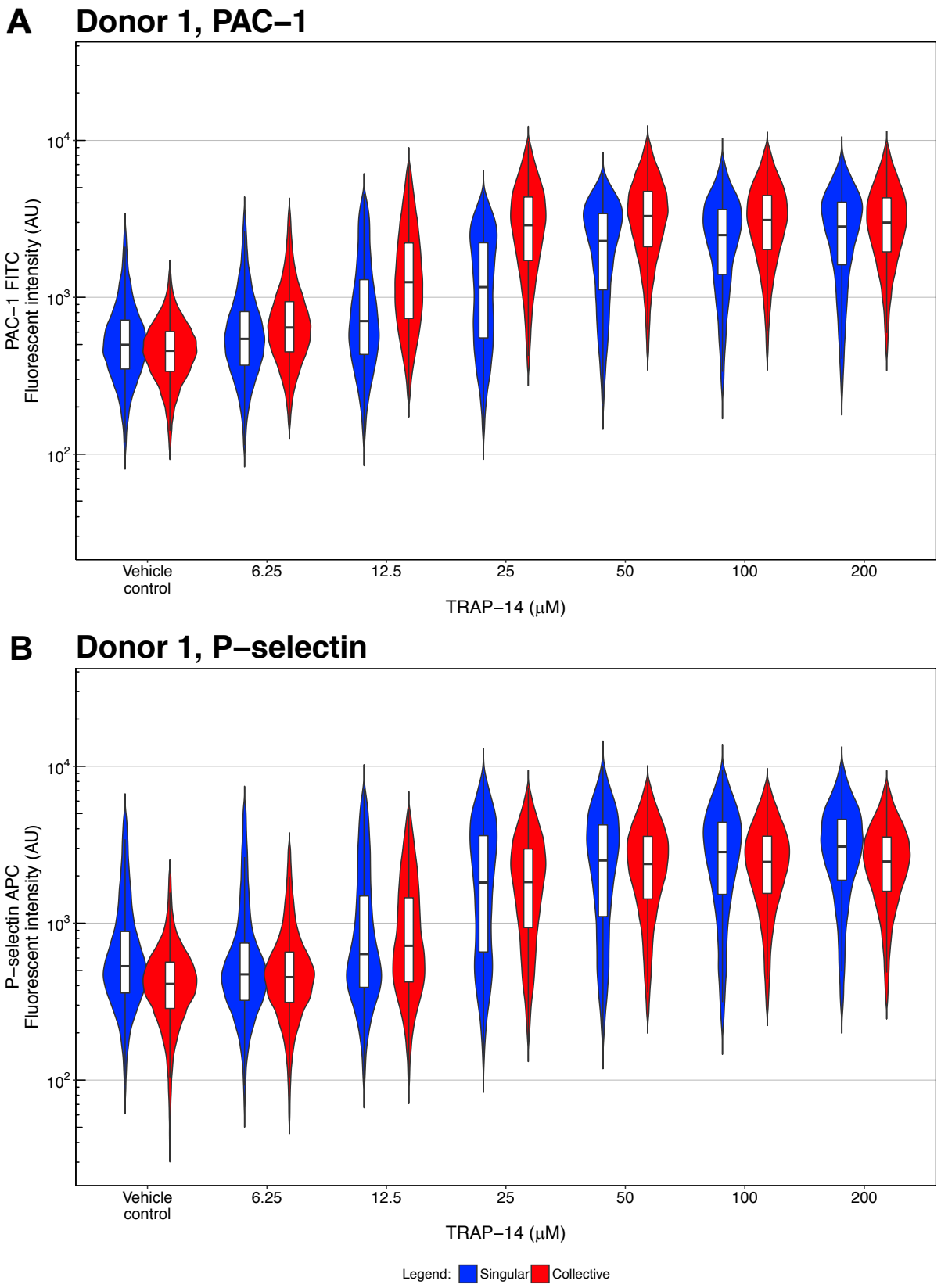


Figure 3-14 Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to TRAP-14 (Thrombin Receptor Activating Peptide) stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. N=2 (also see Figure B-16). Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-13. The RA for singular compared to collective for PAC-1 activation peaks at 25 μM TRAP-14 at 4.2 and for P-selectin expression at 50 μM TRAP-14 at 1.5. Singular RA vehicle compared to TRAP-14 stimulation peaks at 200 μM at 6.0 and 7.0 respectively for PAC-1 and P-selectin, while collective RA peaks at 50 μM at 14 for PAC-1 and at 200 μM 8.5 for P-selectin. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

3.3.7 Trap-14 triggers a small hypersensitive subpopulation

Up to this point all observations were made upon stimulation of platelets with convulxin. However, different agonists cause different platelet responses. Therefore, single platelet function was also studied in response to TRAP-14, a PAR-1 agonist that does not cause fibrinogen cleavage and subsequently eliminates the need for inhibitors of fibrin formation.[162] The dose-response relationship shows a clear increase in activity with increasing dose of TRAP-14 and a clear S curve shape for the collectively stimulated platelets but a less clear shape for the single platelets (Figure B-14). Some platelets never become positive for either PAC-1 or P-selectin (Figure 3-14). There is a slight sensitivity difference between platelets stimulated in the absence or presence of paracrine signalling but much less compared to convulxin stimulation. There is a small subpopulation of single platelets that is between 2 and 4-fold more sensitive than the rest of the single platelets (Figure 3-15). Furthermore, the single platelet response is more binary and less smooth than the collective response (Figure B-15). This response is similar in a second donor (Figure B-16).

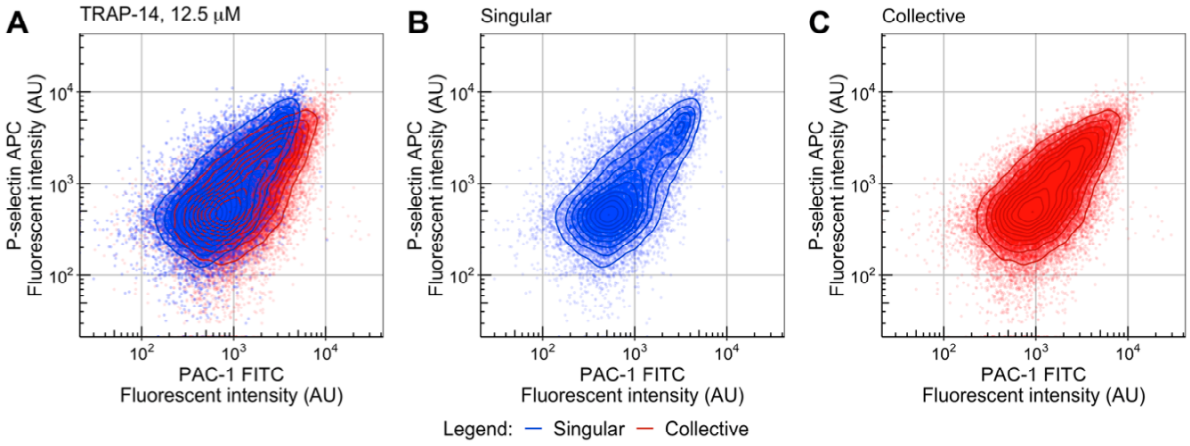


Figure 3-15 Relationship between PAC-1 and P-selectin expression of singular (blue) and collective (red) platelets stimulated with 12.5 µM of TRAP-14. Scatter plot of **A)** overlay of singular and collective platelet response, **B)** only singular and **C)** only collective platelet response. Data is selected from the experiment depicted in Figure 3-14. Data is shown as scatter plot with contour plot showing densities.

3.3.8 A small but very hypersensitive subpopulation is observed when stimulated with ADP

Another agonist frequently used in platelet function studies is ADP. ADP is especially interesting in this study because it is a secretion product, released by degranulation of the dense granules. The dose-response relationship has a S-curve shape for low, medium and high concentrations (0.01-100 µM) but a sharp reduction in activity associated with very high concentrations (1,000 and 10,000 µM) of ADP (Figure B-17). Furthermore, the P-selectin response is limited to approximately half of positive platelets at the peak (100 µM) activation level while most platelets become more positive for PAC-1 (Figure 3-16). There is a slightly reduced sensitivity of single platelets compared to collectively stimulated platelets, but this can only be observed for medium concentrations of ADP (0.1 and 1 µM) for PAC-1 binding and medium-high concentrations (1 and 10 µM) for P-selectin expression. At a concentration of 0.1 µM a small (~15%) hypersensitive platelet subpopulation can be observed (Figure 3-17). This subpopulation is between 10 and 100-fold more sensitive than the rest of the single platelets (only at a concentration of

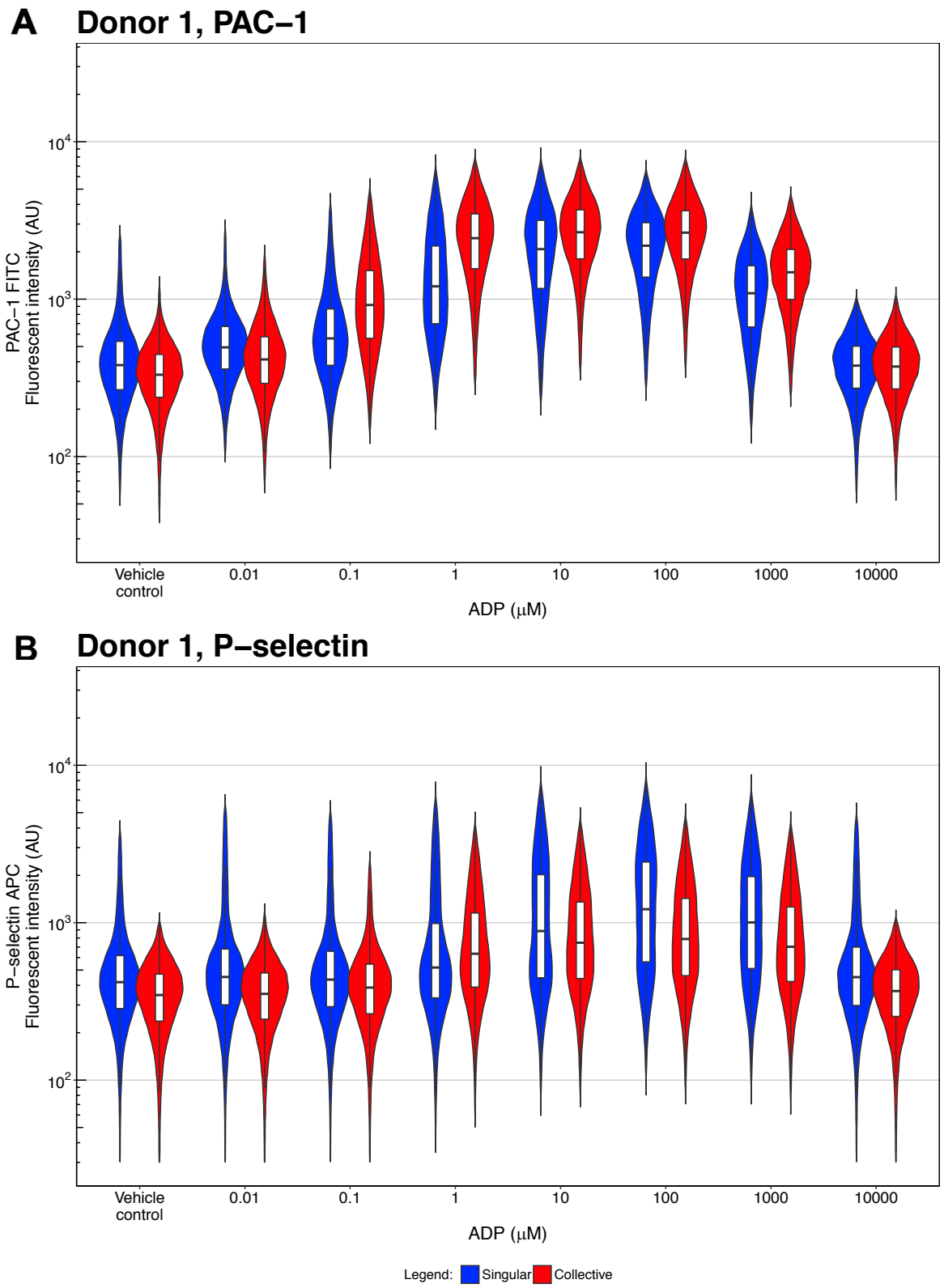


Figure 3-16 Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to ADP (Adenosine DiPhosphate) stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. N=2 (also see Figure B-19). Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-15. The RA for singular compared to collective for PAC-1 activation peaks at 1 μM ADP at 3.5 and for P-selectin expression at 1.1. Singular RA vehicle compared to ADP stimulation peaks at 100 μM at 6.1 and 2.0 respectively for PAC-1 and P-selectin, while collective RA peaks at 100 μM at 13 for PAC-1 and at 1.7 for P-selectin. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

10 µM are most of the single platelets positive for PAC-1). However, the platelet collective sample looks similar although the difference to full activation is smaller (max 10-fold).

The singular platelet response to ADP is more binary than the collective platelet response. This is mostly evident with the P-selectin expression (Figure B-18). The collective response shows a transition phase, most prominent on the PAC-1 binding, that is never observed in singular platelet response. The difference between the two patterns is less clear than upon stimulation with convulxin or TRAP-14. Similar observations can be made for a second blood donor (Figure B-19).

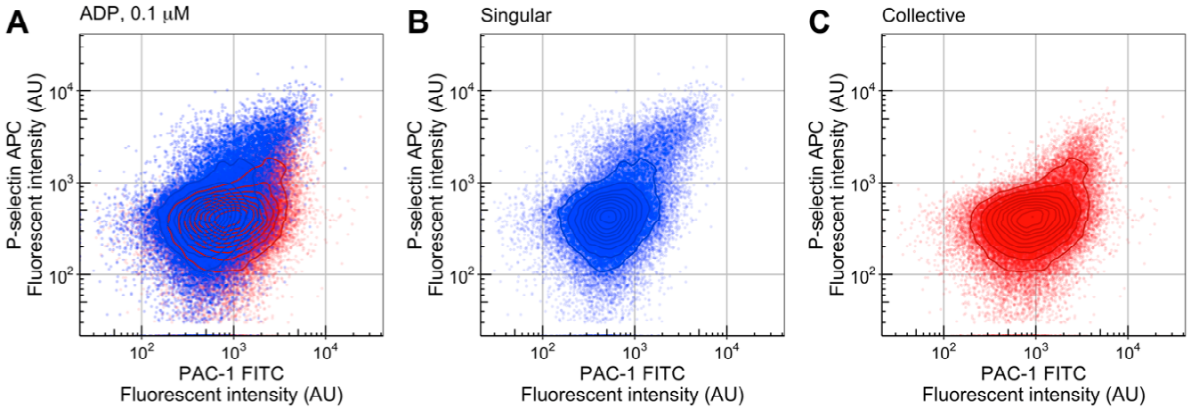


Figure 3-17 Relationship between PAC-1 and P-selectin expression of singular (blue) and collective (red) platelets stimulated with 0.1 µM of ADP. Scatter plot of **A)** overlay of singular and collective platelet response, **B)** only singular and **C)** only collective platelet response. Data is selected from the experiment depicted in Figure 3-16. Data is shown as scatter plot with contour plot showing densities.

3.4 Discussion

In this study we show that single platelets have various sensitivity levels and that in the presence of paracrine signalling system amplification results in a collective higher sensitivity population. Furthermore, a hypersensitive subpopulation that was 10-fold more sensitive (to convulxin) was found in single platelet response. Also, in general the sensitivity of platelets is reduced in the absence of paracrine signalling. Therefore, we have shown the need for functional study of single platelets and expanded the knowledge on platelet subpopulations.

To study single platelets and intrinsic variability between platelets we encapsulated platelets in droplets. A strong sensitivity difference between platelets stimulated in the absence or presence of paracrine signalling was found. However, the best way to test the statistical significance can be debated. The flow cytometry data is not normally distributed in this study and therefore parametric statistics such as ANOVA or T-test are inappropriate. A chi-squared or Fisher exact test would be appropriate to test the difference between positive platelets in two samples. nonetheless, taking every single platelet into account leads to the overpowering of these studies.[163] Moreover, the magnitude of the effect is not taken into account. Using the relative activation with a confidence interval makes it possible to study the magnitude of the response and its significance while minimizing the effect of the overpowered test. Although, this simplifies the platelet response to a binary activity level (active or non-active) and not using the degree of activation that fluorescent intensity brings and can only be used to compare one sample with another. In order to use the 8 donors for a conclusion, we also plotted an Emax model to the dose-response curve. This is a model for dose-response relationships that allows the estimation of EC-50 values where the curve reaches 50% effect. However, with a sample size of only 8 donors the

probability of identifying the right fit model is slim. In literature it is described that this probability for 10 donors is only 0.28.[164] Despite this, the observed difference between EC-50 value is so large that the probability that the right model would not establish a difference at all between the single and collective platelet model is highly unlikely.

Besides the differences between many singular and collectively stimulated platelets, a hypersensitive subpopulation of platelets was found. This was observed for $\alpha_{IIb}\beta_3$ activation as well as both alpha and dense granule secretion after stimulation with convulxin, TRAP-14 and ADP. A hypersensitive population was found for dense granule secretion, even though the dense granule secretion required a higher concentration than alpha granule secretion, which is in accordance with the literature.[59] This study also observed that the α-granule response to ADP was weak. In clinical tests the measurement of lumiaggregometry with ADP is not recommended because granule release upon activation with ADP can be absent in healthy individuals.[70] While this is the other type of granule it is an indication of secretion in general.

In the literature, platelet hypersensitivity is usually described as the overall elevated response observed in donors with elevated thrombosis risk such as diabetes, or in the context of non-responders to antiplatelet treatment.[25, 165] Platelet subpopulations are only described in the context of procoagulant platelets or non-functional differences in platelets.[59] The hypersensitivity of a platelet subpopulation is a novel finding of this study.

The encapsulation of platelets within droplets is the only method currently available to study single platelet function. However, the encapsulation in droplets also restricts the environment of the platelet. This can lead to elevated autocrine signalling, causing platelet activation to become more binary. Encapsulation in bigger droplets decreases this problem but simultaneously increases the costs of the assay. Also, bigger droplets require the platelet suspension to be diluted even further than already necessary with the currently used droplet size. While this study dilutes the platelet suspension with buffer rather than autologous platelet poor plasma this can still interfere with some anti-coagulant factors and disrupt the balance between pro and anti-coagulant stimuli. On the other hand, bigger droplets are easier to handle and stabilize. Another potential problem with the smaller droplets is that lower concentrations of big signalling molecules can follow a Poisson distribution causing variations in available molecules per platelet. Finally, the encapsulation of platelets is labour intensive which greatly limits the number of samples that can be measured within the recommended life span of platelets in suspension, 4 hours after blood draw.[154] This study has limited the sample stimulation to 6 hours after blood draw but delayed stimulation of platelets might have an effect on the measured response. In some cases, the measurement of more than 8-10 samples per donor would be advantageous but is not possible with this method. Each sample takes about 20-30 minutes to stimulate within the droplets and the platelet preparation takes approximately 90 minutes. This makes adding more samples while staying within a 4-6 hour limit, between blood draw and measurement, not practically possible without devices in parallel (which is not feasible because of lab footprint of setup and costs).

However, this method can make general and useful contribution to the understanding of platelet function and thrombus formation. Encapsulating higher concentrations of platelets in the bigger droplets already used (Ø50 µm) allows the study of finite platelet collections to study the aggregatory capabilities while limiting paracrine signalling to the small number of platelets within a droplet (5-15 platelets per droplet). Preliminary results show aggregation at levels comparable with the samples stimulated in presence of paracrine signalling described in this study (Figure B-20). Furthermore, this method can be used to

elucidate some of the potential sources of platelet variety such as age and receptor density. In clinical studies this method can be used to find the clinical relevance of hypersensitive subpopulations in hyper-response phenotypes such as diabetes but also hypo-response to anti-platelet therapies.

Understanding the variability of platelets and their role in thrombus formation could lead to the development of new targets and new approaches for anti-platelet therapies. Furthermore, identifying individuals with more hypersensitive platelets could potentially improve the prognostic and diagnostic occurrence of thrombosis.

Chapter 4 Intrinsic heterogeneity of platelet procoagulant response

4.1 Introduction

The most widely studied subpopulation of platelets is the procoagulant platelet phenotype.[59] This population was initially named COAT (COllagen And Thrombin activated) platelets by Alberio, Dale et al in 2000[45] because of their origin in activation with high concentrations of collagen and thrombin. This phenotype has an inactivated $\alpha_{IIb}\beta_3$ receptor and a strong procoagulant, phosphatidylserine (PS) exposure on the outer membrane.[33, 45, 48, 49, 52] The maximum percentage of platelets of this phenotype is 80%.[52]

From studies into this heterogeneity and others, two leading causes of functional (or response) heterogeneity have been proposed, namely intrinsic heterogeneity and spatiotemporal (extrinsic) heterogeneity. The first refers to the capacity of platelets to respond to stimuli such as heterogeneity caused by for example megakaryocyte heterogeneity, megakaryocyte production of platelets and platelet age. The second describes the response heterogeneity from platelets that have similar capacity for response to stimuli but respond differently because of their specific place and time of inclusion in a growing thrombus. With parallel-plate flow chambers it has been shown that procoagulant platelets are mainly at the outside of a thrombus while proaggregatory platelets are more closely located to the core of the thrombus.[2, 88, 166, 167] This suggests that a potential source of heterogeneity lies in the position within the growing thrombus. However, this difference between intrinsic and spatiotemporal heterogeneity cannot be measured unless you can fully exclude one. By encapsulating platelets in droplets, they are isolated from the influence of other platelets in the vicinity and truly intrinsic variation in platelet response can be studied. Therefore, this study aims to adapt a droplet microfluidics approach to study platelet function.

4.2 Methods

4.2.1 Monoclonal antibodies and reagents

Fluorescent staining was achieved with fluorescein isothiocyanate (FITC) conjugated PAC-1 (PAC-1 clone), FITC conjugated anti-CD63 (H5C6 clone), FITC and allophycocyanin (APC) conjugated anti-CD62P (P-selectin) (AK-4 clone), APC conjugated Annexin V, R-phycoerythrin (PE) conjugated anti-CD42b (HIP1 clone) and PE conjugated anti-CD61 (VI-PL2 clone) obtained from Becton Dickinson Pharmingen (San Jose, California, United States). Platelets were activated with convulxin, a snake venom toxin specifically activating the GPVI receptor (main collagen receptor) obtained from Enzo Life Sciences (Exeter, United Kingdom) and thrombin from Sigma Aldrich (Sigma Aldrich, St. Louis, Missouri, United States). Coagulation was inhibited with Rivaroxaban (factor Xa inhibitor) from Advanced ChemBlocks Inc. (Burlingame, California, United States) and H-Gly-Pro-Arg-Pro-OH (GPRP) from Bachem AG (Bubendorf, Switzerland). Blood samples were diluted in HEPES buffer containing 136 mM NaCl, 2.7 mM KCl, 10 mM HEPES and 2 mM $MgCl_2$

(pH 7.45), stored at 4-7 °C, with glucose 0.1% w/v and BSA 1% w/v added freshly before use. Agonists and antibodies were diluted in HEPES buffer with 2.5 mM $CaCl_2$ (final concentration with platelet suspension added was 2 mM $CaCl_2$).

4.2.2 Participants

Blood was obtained by venepuncture from healthy volunteers after obtaining written informed consent. Ethics approval was obtained from the NRES Committee South Central – Hampshire B (REC reference 14/SC/0211) and from ERGO (protocol number 5538). This study used blood from 2 males, one between 30-40 years and one between 40-50 years of age. The blood was collected using a 21 G needle, into Vacuette tubes containing 1:10 v/v 3.2% trisodium citrate (0.109 M) and gently inverted three times. The first 4 mL was collected into a K_2EDTA containing Vacutainer® tube (BD, Franklin Lakes, New Jersey, United States) and only used for platelet counts.

4.2.3 Blood preparation

Directly after blood collection, citrate tubes were centrifuged at 240 g for 15 min without a brake to prepare platelet rich plasma (PRP). The PRP was set aside for approximately half an hour before use. In the meantime, the platelet count was determined (see section 4.2.4). Subsequently, platelet counts in PRP were adjusted to $100x10^6$/mL using HEPES buffer and the platelet count measured using the same protocol. This platelet count adjusted PRP was added to a solution of Rivaroxaban (final concentration 0.5 µM and 0.05% DMSO) and (in cases where thrombin was used) GPRP (final concentration 100 mM) diluted in HEPES buffer to adjust the count to the final concentration of $25x10^6$/mL. After incubation of 15 minutes this platelet suspension was loaded in the syringe or used for controls.

4.2.4 Platelet counting

The platelet count in the PRP and EDTA anti-coagulated whole blood was determined using a protocol by Masters and Harrison, 2014.[147] Briefly, 20 µL of a 20-fold diluted PRP (in HEPES buffer) was stained for 15 min with 1 µL anti-CD61. After the incubation time, this stained sample was further diluted 50-fold. An Accuri C6 flow cytometer (BD Biosciences, Franklin Lakes, New Jersey, United States) was used to analyse 100 µL of this diluted sample and platelet counts were calculated from the number of positive events.

4.2.5 Droplet generation

Droplets were generated using a PDMS droplet formation device with inlets for the oil-surfactant mixture, agonist, antibody and platelet solution (Figure 3-1). For droplet generation, fluorinated, gas-permeable oil and surfactant were used, especially designed for live cell assays, 0.75% v/v 008-Fluorosurfactant (Ran biotechnologies, Beverly, Massachusetts, United States) in HFE - 7500 (3M™ Novec™ 7500 Engineered Fluid). Fine bore polythene, medical grade and sterile, tubing with an outer diameter of 1.09 mm and an inner diameter of 0.38 mm (Smiths Medical International, Hythe, United Kingdom) was plugged into the inlets (no separate bonding procedures were used). On the other end, the tubing was pulled over a 25 G needle and connected to a syringe. Pulse free syringe pumps (Fusion 200, Chemyx, Stafford, Texas, United States) were used to infuse the solutions into the devices. Solutions were infused into the devices by pulse free syringe pumps, at speeds

of 1200 µL/hr oil, 120 µL/hr agonist, 120 µL/hr antibody and 60 µL/hr diluted platelet solution, to a total flow rate of 1500 µL/hr (flow ratio of aqueous to oil of 1:4, agonist to antibody of 1:1 and platelets to agonist/antibody of 1:4). Live droplet formation was monitored and recorded using a high speed (2500 frames/sec) camera (Vision Research, Wayne, New Jersey, United States) and dedicated software (Phantom Camera Control Software, Vision Research).

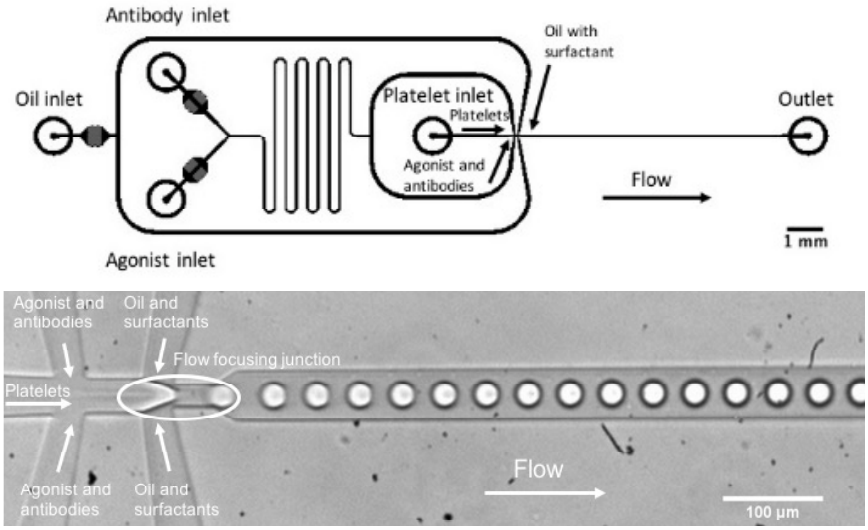


Figure 4-1 Device used to study single platelet function in response to an agonist. The junction where platelets are mixed with the antibody-agonist mixture is 90 µm long and 35 µm wide. This suspension is mixed with oil and surfactant and forced through a pinch section, resulting in droplet formation. Platelets are in the junction for ~0.8 msec and on the chip outside droplets for ~230 msec. After encapsulation the droplets travel along a straight channel for 7 mm (~17 msec) to stabilise the droplet interface.

4.2.6 Flow cytometry

Droplets were collected for 5 min continuously and incubated in the dark for 10 min to ensure all platelets were incubated between 10 and 15 min which was determined to be the optimum incubation time. Afterwards 200 µL fixative and 200 µL 1H,1H,2H,2H-perfluoro-1-octanol (Sigma Aldrich, St. Louis, Missouri, United States) was added to destabilise the droplet interface. After a few minutes the supernatant was taken off and measured immediately on the flow cytometer (Accuri C6 flow cytometer from BD Biosciences).

Collective samples (platelet suspension in tube, no oil or droplets) were stimulated in the same ratios as the droplet generation runs (agonist to antibody of 1:1 and platelets to agonist/antibody of 1:4), 10 µL agonist, 10 µL antibody and 5 µL of platelet solution. After 15 min of adding the platelet solution, 100 µL of fixative is added to stop further platelet activation and antibody binding. Samples were incubated in the fridge and measured within 4 days. Before cytometry, 100 µL of PBS was added to dilute the platelets.

Table 4-1 The antibody panels used by this study and their respective concentrations.

		Conjugated with		
		FITC	PE	APC
Antibody panel	A	PAC-1 1.25 ng/µL	CD42b 1.25 ng/µL	P-selectin 0.63 ng/µL
	B	PAC-1 1.25 ng/µL	CD42b 1.25 ng/µL	Annexin V 0.08 ng/µL
	C	P-selectin 2 ng/µL	CD61 0.25 ng/µL	Annexin V 0.08 ng/µL
	D	CD63 2 ng/µL	CD61 0.25 ng/µL	P-selectin 0.63 ng/µL

4.2.7 Statistical analysis

Droplet videos were analysed using ImageJ software (open source[161]). Flow cytometry samples were analysed with FLOWJO (Ashland, Oregon, United States). Graphs and statistical analysis were performed using GraphPad Software (Prism) (San Diego, California, United States) or R (open source). Significance testing of single platelet experiments was performed with the relative risk / risk ratio procedure plus confidence intervals. For these single platelet experiments this test was named relative activation.

Platelets were selected with a gate on the CD42b or CD61 antibody fluorescence intensity and subsequently doublets were removed with gates on the forward scatter area vs height and forward scatter height vs width. Lastly a gate was placed on the forward scatter height vs sideward scatter height to gate out non-platelet sized events (see Chapter 2). Collective samples were performed in triplicate and subsequently a representative sample was selected. This selection was based on the median of triplicate values and the sample which was most often the median in 7 parameters (FSC-A, FSC-H, SSC-H, FL1-H, FL2-H and FL4-H) was selected. Ties were broken with the median of the FL1-H parameter.

4.3 Results

4.3.1 Loss of membrane asymmetry is triggered by high concentrations of convulxin

Resting platelets have membrane phospholipid asymmetry with phosphatidylserine only present on the inner leaflet of the plasma membrane. Upon activation, an influx of cytosolic calcium activates TMEM16F which catalyses phospholipid transport leading to a loss in phospholipid asymmetry and phosphatidylserine exposure.[22] This phosphatidylserine (PS) exposure can be detected with the binding protein Annexin V. To test whether this mechanism is present in single platelets, platelets were encapsulated in droplets and stimulated with convulxin. Because the TMEM16F activation is calcium dependent, which is chelated by citrate in the anticoagulant used to collect blood, the agonist and antibody were diluted using HEPES buffer with $CaCl_2$ (final concentration 2 mM). However, this also causes conversion of prothrombin into thrombin by factor Xa and thrombin is a potent platelet agonist itself. To prevent the thrombin formation, the platelet suspension was pre-incubated with Rivaroxaban, a factor Xa inhibitor (see chapter 2). The dose-response curve of platelet stimulation with convulxin in presence of paracrine signalling shows an initial s-curve increase in PAC-1 binding (Figure 4-2). This is followed by a decrease in PAC-1 binding and coincident increase in Annexin V binding. The pattern of activation is similar for single platelets although higher concentrations (approximately 10 times higher) are required for a similar increase in PAC-1 binding. The response to the highest concentration (100 ng/mL) of convulxin is similar however. The total number of platelets with PS exposure remains low at around 20-25% for both singularly and collectively stimulated platelets. Similar observations can be made for the other blood donor in this study (Figure C-3).

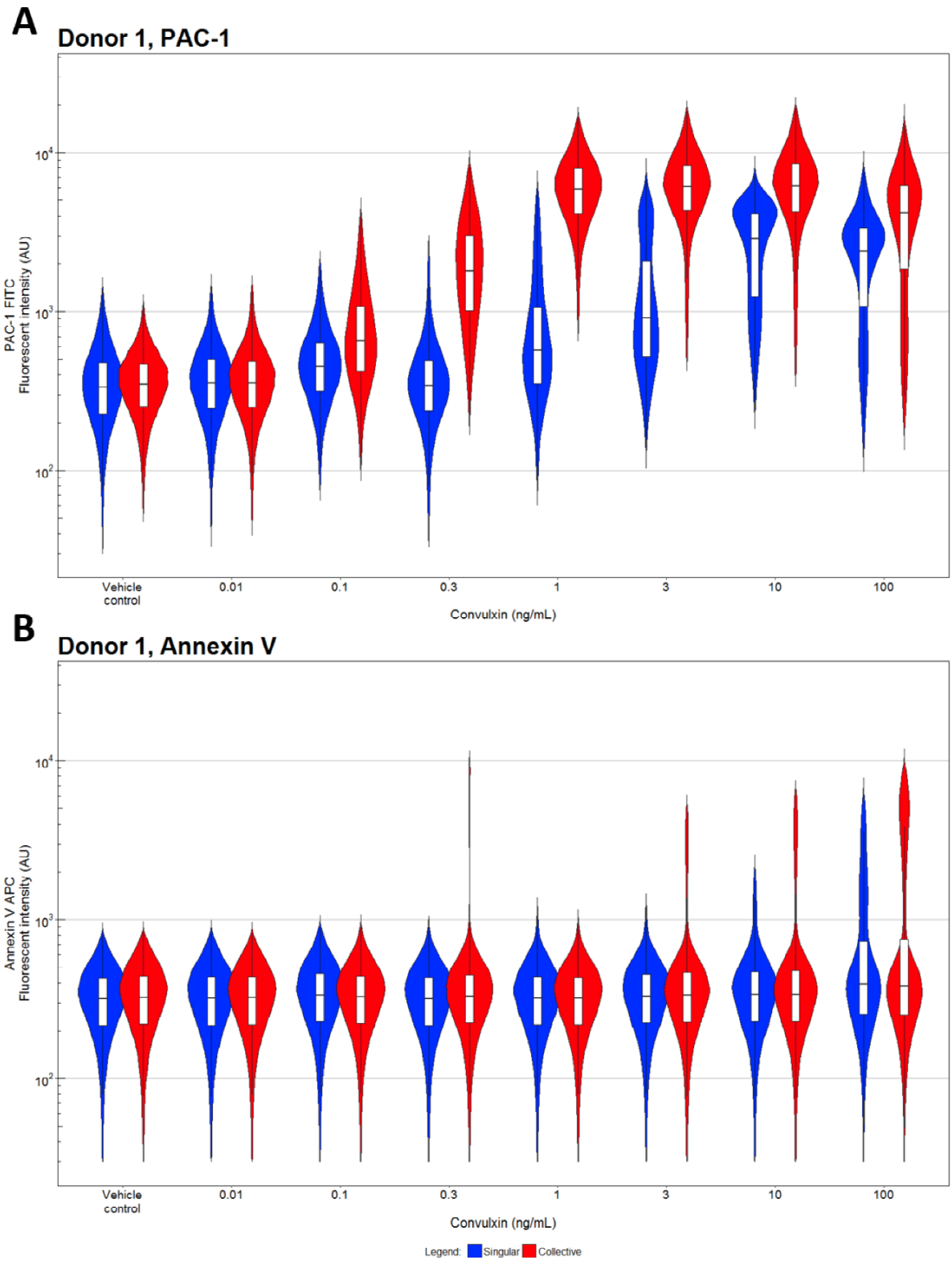


Figure 4-2 Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 antibody or **B)** Annexin V binding in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelet stimulation in the presence of $CaCl_2$ (2 mM) and Rivaroxaban (0.1 μM in 0.01% DMSO). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile, tails are not trimmed. N=2 (also see Figure C-3). Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-1. The RA for singular compared to collective for PAC-1 activation peaks at 1 ng/mL convulxin at 47. Singular RA vehicle compared to convulxin stimulation peaks at 10 ng/mL at 4.7, while collective RA peaks at 1 ng/mL at 63. The RA for Annexin V binding were all <2 including singular compared to collective and vehicle compared to convulxin.

4.3.2 PAC-1 and Annexin V binding are mutually exclusive

When looking more closely at the response of platelets to 100 ng/mL in the presence of $CaCl_2$ a distinct pattern of activation can be observed (Figure 4-3). Two clear populations can be observed, one that is positive for PAC-1 and negative for Annexin V, and one which is positive for Annexin V while negative for PAC-1. Only a few platelets are either negative or positive for both PAC-1 and Annexin V. This heterogeneous response can be observed with both singularly and collectively stimulated platelets. However, singular platelets positive for Annexin V have a slightly lower fluorescent intensity of Annexin V signal compared to collective platelets.

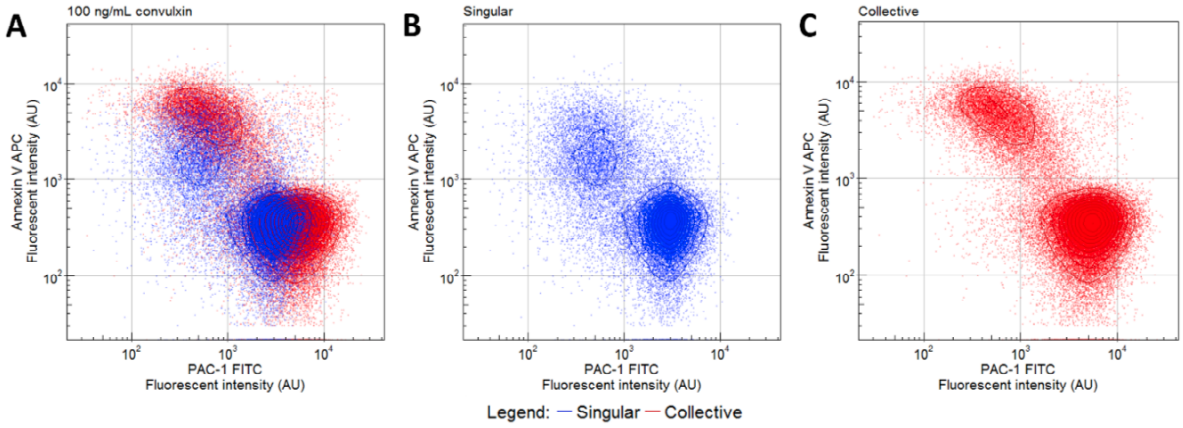


Figure 4-3 Relationship between PAC-1 and Annexin V binding of singular (blue) and collective (red) platelets stimulated with 100 ng/mL of convulxin. Scatter plot of **A)** overlay of singular and collective platelet response, **B)** only singular and **C)** only collective platelet response. Data is selected from the experiment depicted in Figure 4-2. Data is shown as scatter plot with contour plot showing densities. The procoagulant platelet population consists of 16% of singlular platelets and 17% of collective platelets.

4.3.3 Convulxin and thrombin stimulations causes two populations of single platelets

It has been suggested in the literature that dual agonist stimulation (with both convulxin and thrombin) of platelets is more effective in the formation of the PS-exposing, procoagulant platelets (also called COAT or coated platelets) than convulxin alone.[33, 45, 49] To study this effect, platelets were stimulated with 300 ng/mL of convulxin and 3 U/mL of thrombin (similar concentration as found in literature) and a dose response curve was made from there by serial dilution. The dual agonist stimulation dose response curve shows an initial increase in PAC-1 binding (Figure 4-4). This initial increase is similar to that observed with convulxin only for both singularly and collectively stimulated platelets (Figure 4-2). Nevertheless, the addition of thrombin leads to a loss of PAC-1 binding at lower concentrations of convulxin than with convulxin alone. The dual agonist stimulated platelets are at their peak of PAC-1 binding with 3 ng/mL/0.3 U/mL stimulation for both singularly and collectively stimulated platelets before decreasing while the convulxin only still increases for the singularly and is stable for the collectively stimulated platelets. The first population of Annexin V positive platelets can be observed at concentrations of 10 ng/mL with or without added thrombin. The addition of thrombin does approximately double this population (from 4.5 to 8.7% for singular platelets and 8.7 to 18% for collective platelets). Thrombin stimulation alone leads to the first population of PAC-1 positive

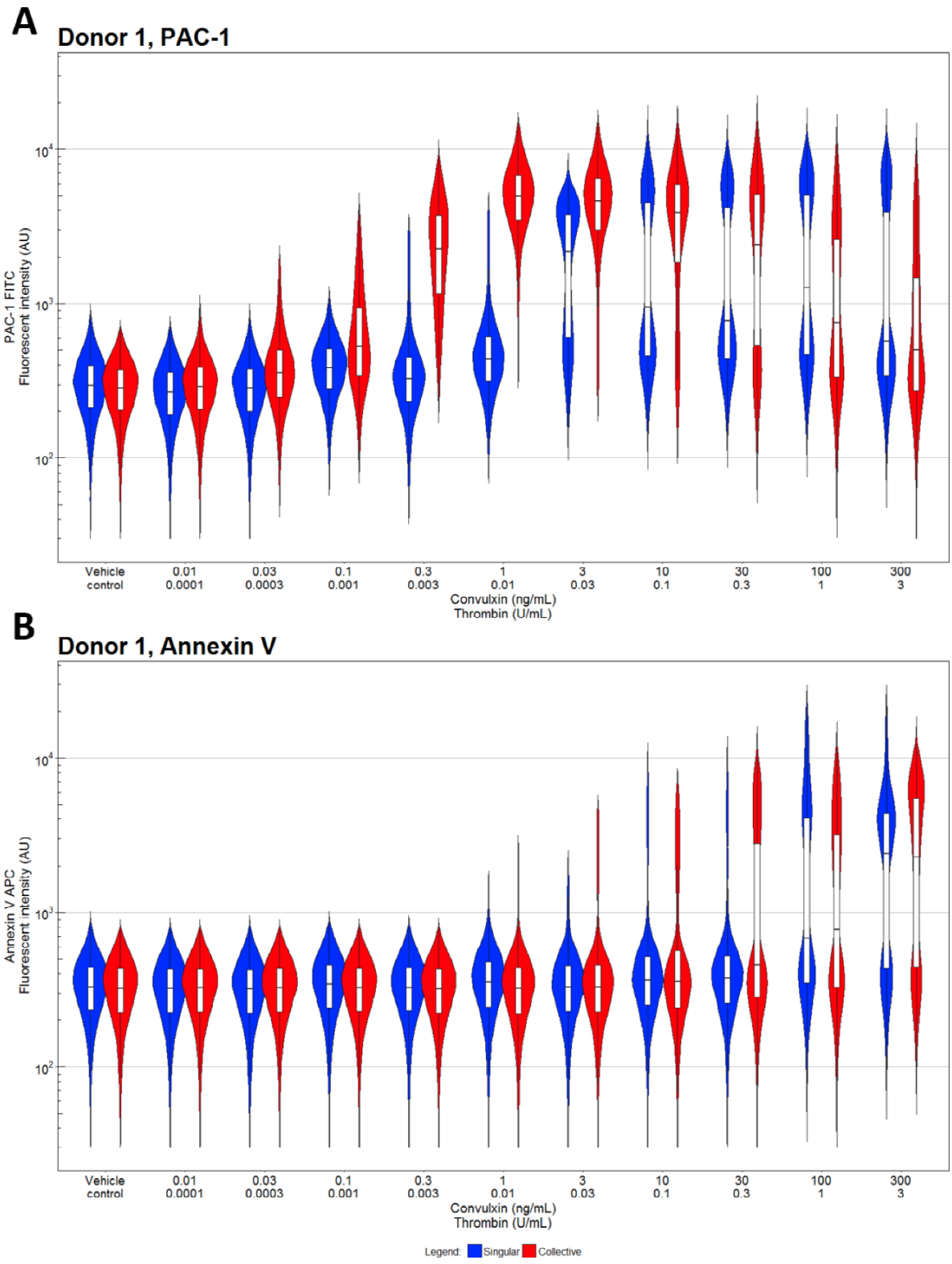


Figure 4-4 Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 antibody or **B)** Annexin V binding in response to dual agonist stimulation with convulxin and thrombin in droplets (singular, blue) or in suspension (collective, red). Platelet stimulation in the presence of $CaCl_2$ (2 mM), GPRP (20 mM) and Rivaroxaban (0.1 μM in 0.01% DMSO). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile, tails are not trimmed. N=2 (also see Figure C-6). Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-3. The RA for singular compared to collective for PAC-1 activation peaks at 1 ng/mL convulxin and 0.01 U/mL thrombin at 26. Singular RA vehicle compared to dual agonist stimulation peaks at 100 ng/mL and 1 U/mL at 2.7, while collective RA peaks at 1 ng/mL and 0.01 U/mL at 28. The RA for Annexin V binding were all <2 including singular compared to collective and vehicle compared to convulxin, except 300 ng/mL convulxin and 3 U/mL thrombin wich was 2.5 for singular and 2.6 for collective stimulation.

platelets to be observed at 0.1 U/mL and maximum PAC-1 at 1 U/mL and higher (Figure C-5). No Annexin V binding can be observed at all with thrombin stimulation up to 3 U/mL. Similar observations can be made with the other donor (Figure C-6-Figure C-8).

4.3.4 Medium concentrations of convulxin/thrombin stimulation leads to all platelets binding PAC-1

Stimulation of platelets in suspension (collective) with the highest concentration of 300 ng/mL of convulxin and 3 U/mL of thrombin resulted in the majority (~70%) of platelets being negative for PAC-1 and positive for Annexin V (Figure 4-5). The same stimulation of single platelets resulted in slightly lower amount (~60%) of platelets that are Annexin V positive.

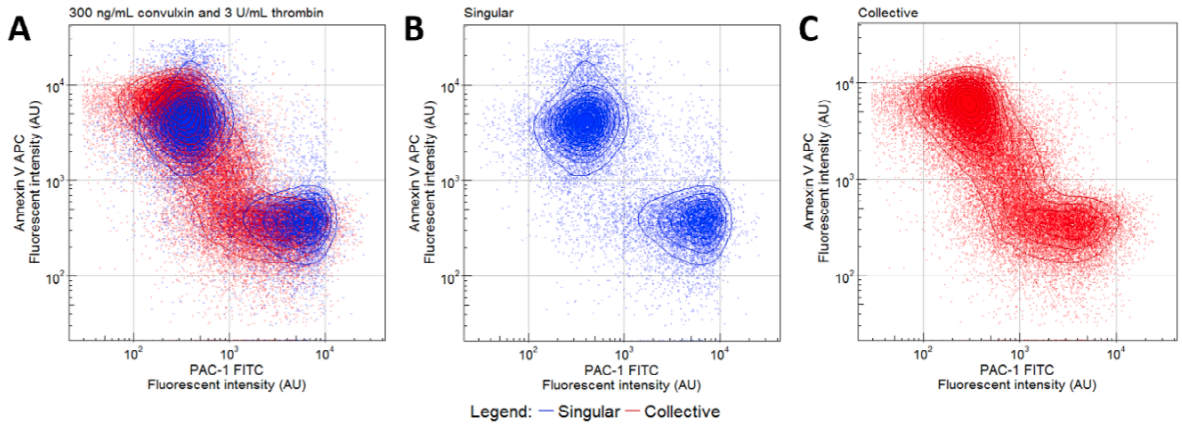


Figure 4-5 Relationship between PAC-1 and Annexin V binding of singular and collective platelets stimulated with 300 ng/mL of convulxin and 3 U/mL of thrombin. Scatter plot of **A)** overlay of singular and collective platelet response, **B)** only singular and **C)** only collective platelet response. Data is selected from the experiment depicted in Figure 4-4. Data is shown as scatter plot with contour plot showing densities. The procoagulant platelet population consists of 57% of singlular platelets and 58% of collective platelets.

Lower concentrations (convulxin 1 ng/mL/thrombin 0.01 U/mL and 3 ng/mL/0.03 U/mL) lead to over 90% of collectively stimulated platelets to being positive for PAC-1 after which the amount of PAC-1 decreases (Figure 4-6). However, the singularly stimulated platelets reach the maximum of PAC-1 platelets around the same concentration (3 ng/mL/0.03 U/mL) while only ~60% of platelets are positive for PAC-1. This indicates that the initial PAC-1 binding is sensitive to the sensitivity difference between singular and collective platelets (discussed in chapter 3) while the Annexin V binding and coincident decrease in PAC-1 binding is not.

While virtually all platelets collectively stimulated become positive for PAC-1 before the procoagulant population emerges, the singularly stimulated platelets have a different pattern of activation. When stimulating single platelets with 100 ng/mL of convulxin and 1 U/mL thrombin three populations of activity can be observed (Figure 4-7). A population that is negative for PAC-1 and Annexin V, a population positive for PAC-1 and negative for Annexin V and a population positive for Annexin V and negative for PAC-1. Furthermore, there seem to be some platelets that are (weakly) positive for Annexin V while positive for PAC-1. This activation pattern is never observed with collectively stimulated platelets (all concentrations are shown in Figure C-9) but is observed in the other donor (Figure C-10).

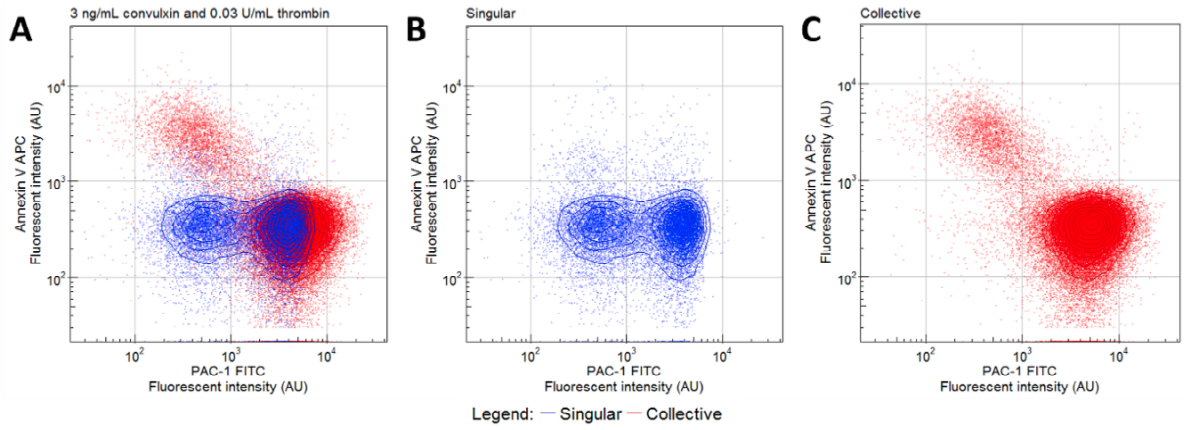


Figure 4-6 Relationship between PAC-1 and Annexin V binding of singular and collective platelets stimulated with 3 ng/mL of convulxin and 0.03 U/mL of thrombin. Scatter plot of **A)** overlay of singular and collective platelet response, **B)** only singular and **C)** only collective platelet response. Data is selected from the experiment depicted in Figure 4-4. Data is shown as scatter plot with contour plot showing densities. The procoagulant platelet population consists of 2% of singlular platelets and 7% of collective platelets.

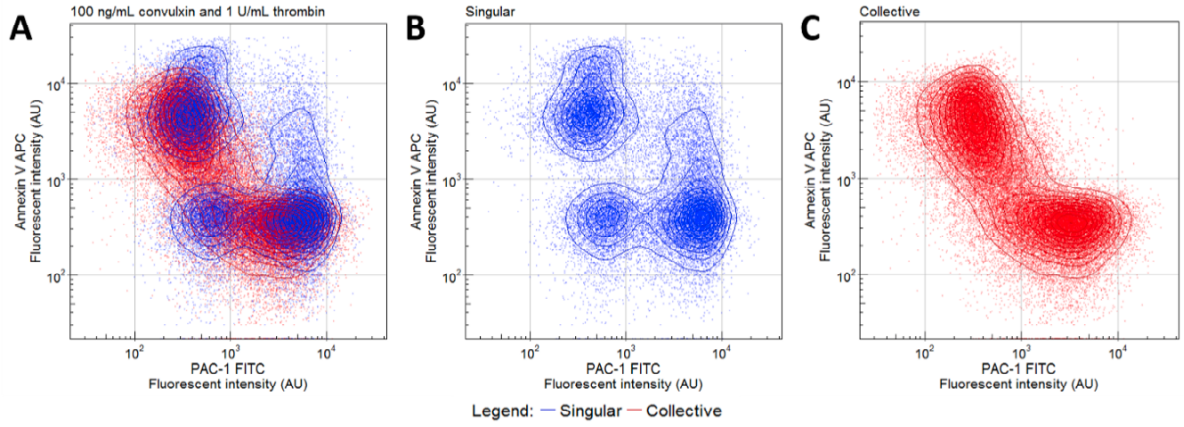


Figure 4-7 Relationship between PAC-1 and Annexin V binding of singular and collective platelets stimulated with 100 ng/mL of convulxin and 1 U/mL of thrombin. Scatter plot of **A)** overlay of singular and collective platelet response, **B)** only singular and **C)** only collective platelet response. Data is selected from the experiment depicted in Figure 4-4. Data is shown as scatter plot with contour plot showing densities. The procoagulant population consists of 32% of singlular platelets and 47% of collective platelets. The proaggregatory population consists of 53% of singlular platelets while 15% of singular platelets is non-active.

4.3.5 Platelets are intrinsically heterogeneous in procoagulant ability

To study the characteristics of heterogeneous population in singularly stimulated platelets with dual agonist, several antibodies were used (Table 4-1). Stimulation with 100 ng/mL and 0.1 U/mL of convulxin and thrombin respectively was used for both singular and collective platelets while collective platelets were also stimulated with no agonists (vehicle control), only convulxin and only thrombin (Figure C-11). Heterogeneity is only observed with PAC-1 and Annexin V binding, not with anti-CD63 or anti-P-selectin binding (Figure 4-8). Singular platelets show similar characteristics of the heterogeneous population compared to collective platelets. Most platelets are positive for P-selectin and CD63 while the PAC-1 and Annexin V binding are split into two populations. The singular platelet heterogeneity seems a bit more profound than the collective response with less platelet outside of the two populations. Stimulation with only thrombin caused near-full activation levels observed with PAC-1, P-selectin and CD63 but no Annexin V binding at all (Figure C-11). Convulxin stimulation only induced the same two populations as triggered by dual agonist stimulation but a smaller Annexin V positive (procoagulant) population.

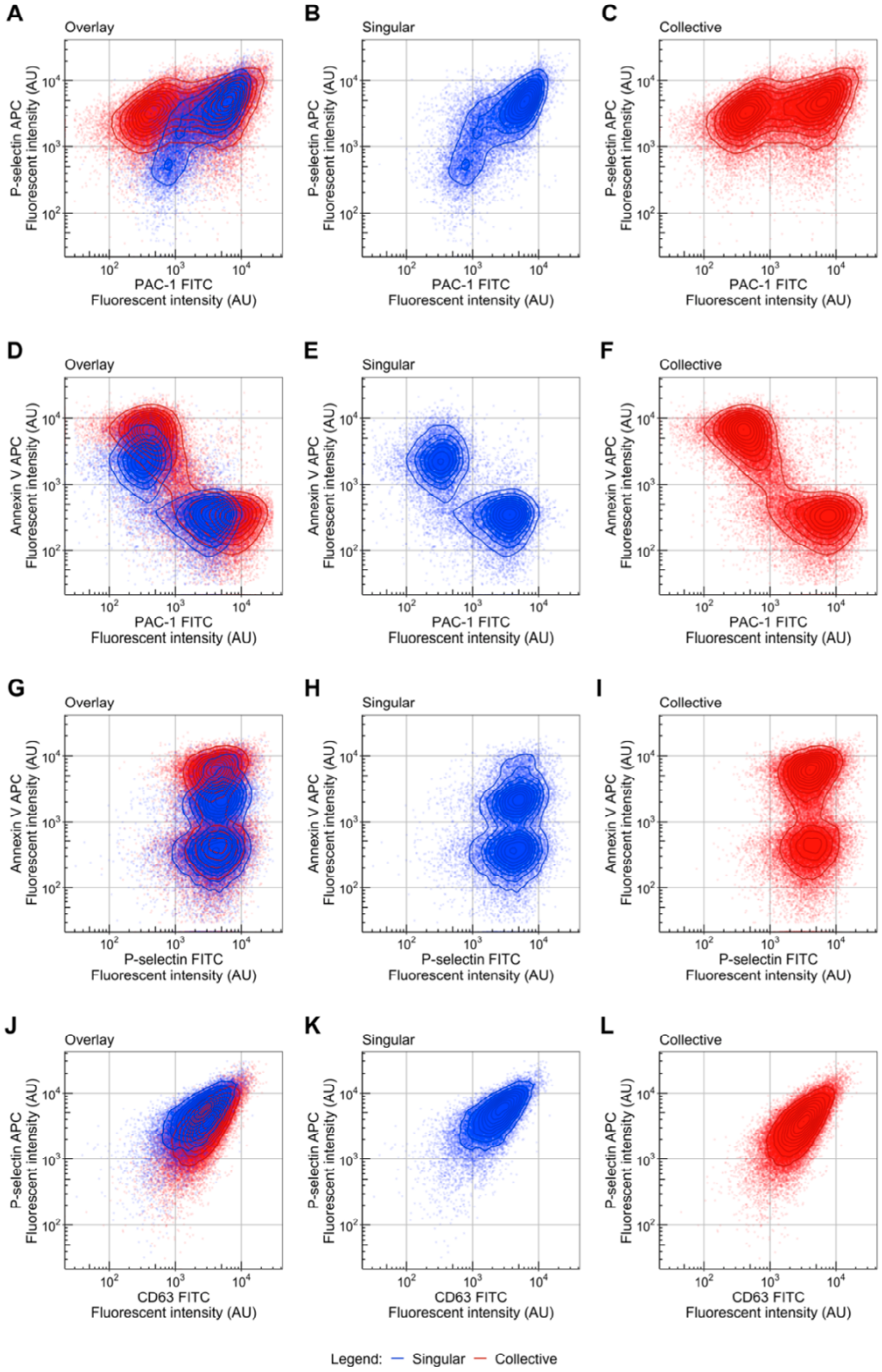


Figure 4-8 Effect of stimulation of singular (blue) and collective (red) platelets with 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Platelet stimulation in the presence of $CaCl_2$ (2 mM), GPRP (20 mM) and Rivaroxaban (0.1 μM in 0.01% DMSO). Platelets were selected with anti-CD42b (A-F) or anti-CD61 (G-L) and following the gating procedure described in section 2.3.5. Scatter plot PAC-1 and P-selectin response (panel A) of **A)** overlay of singular and collective platelet response, **B)** only singular and **C)** only collective platelet response. Scatter plot PAC-1 and Annexin V response (panel B) of **D)** overlay of singular and collective platelet response, **E)** only singular and **F)** only collective platelet response. Scatter plot P-selectin and Annexin V response (panel C) of **G)** overlay of singular and collective platelet response, **H)** only singular and **I)** only collective platelet response. Scatter plot CD63 and P-selectin response (panel D) of **J)** overlay of singular and collective platelet response, **K)** only singular and **L)** only collective platelet response. Data is shown as scatter plot with contour plot showing densities. N=2 (also see Figure C-12). Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-9. The biggest differences of singular vs collective are P-selectin activation (RA of 3.4, where collective platelets have a higher activation level) and PAC-1 (RA of 0.4, where singular platelets have a higher activation level).

4.3.6 Procoagulant platelets have a lower forward scatter

The procoagulant ability of platelets is associated with ballooning and microvesicle formation.[50, 168] While ballooning would increase the size, microvesicle formation and membrane blebbing would decrease the size. To see the effect of the procoagulant platelets on the size, the forward- and sideward scatter was used. Flow cytometry forward scatter can be used as an indication of the size of an event while sideward scatter gives an indication of the granularity. When the procoagulant (Annexin V positive) and proaggregatory (PAC-1 positive) platelets were compared on their scatter pattern upon stimulation with 100 ng/mL of convulxin and 0.1 U/mL thrombin, a marked difference in both forward and sideward scatter is visible. The collective platelets show a marked decrease in size of the procoagulant population compared to the proaggregatory population and more narrow distribution of forward scatter. The singular platelets showed this as well however also had a decreased sideward scatter and tighter distribution of sideward scatter. Whether is caused by the tendency of smaller platelets to become procoagulant or by a decrease in size by switching to a procoagulant state is unknown. However, the narrower distribution suggests that the latter is more likely. This would also suggest that membrane blebbing and microvesicle formation is more prominent than ballooning in this suspension based assay.

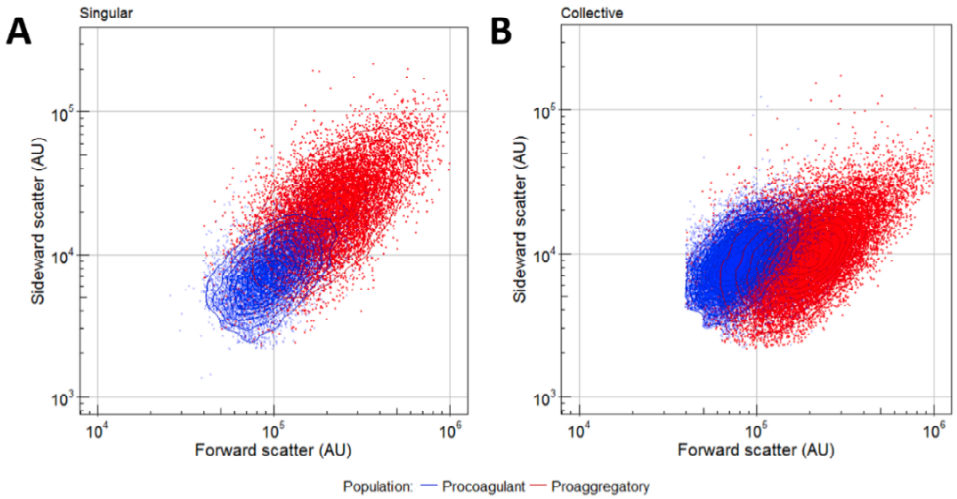


Figure 4-9 Comparing the procoagulant (Annexin V positive, PAC-1 negative) platelets with the proaggregatory (Annexin V negative, PAC-1 positive) platelets on basis of forward scatter (an indication of size) and sideward scatter (an indication of granularity). Platelets stimulated A) singularly and B) collectively. Data is the same as shown in Figure 4-8D-F (panel B), stimulated with 100 ng/mL convulxin and 0.1 U/mL thrombin. A fluorescent intensity >1000 AU for both PAC-1 and Annexin V was used to determine positive platelets. Data is shown as scatter plots with contour plots showing densities.

4.4 Discussion

Functional heterogeneity of platelets has never before been shown to be intrinsic. This is mainly caused by the fact that studying platelets in isolation was not possible before the development of a droplet microfluidic technique and platelet function is a complicated process that is highly dependent on paracrine signalling and spatiotemporal effects. The development of a single platelet method combining droplet microfluidics and flow cytometry has provided the opportunity to study the intrinsic aspects of procoagulant platelet heterogeneity.

This study found that PS exposure and $\alpha_{IIb}\beta_3$ activity form two mutually exclusive populations and that this is (mostly) intrinsic. Furthermore, lower concentrations of stimulation cause full activation of PAC-1 before the PAC-1 binding decreases. This would be in accordance with the deactivation of the $\alpha_{IIb}\beta_3$ receptor rather than no activation.[19, 51] Interestingly, it would seem that the initial PAC-1 increase ($\alpha_{IIb}\beta_3$ activation) is more susceptible to sensitivity changes of single compared to collective platelets than the deactivation of $\alpha_{IIb}\beta_3$ and associated PS-exposure. Another interesting difference between single and collective platelets was the finding that the sideward scatter decreases in procoagulant single platelets but not as prominent in procoagulant collective platelets. This could potentially be because of the enhanced autocrine signalling in the droplets that lead to more degranulation or packing of granules within microvesicles. However, with current results this remains unknown and needs to be researched further.

The literature describes coated platelets or procoagulant platelets as having $\alpha_{IIb}\beta_3$ deactivation, PS-exposure and binding of coagulation factors.[33, 45] Moreover, that PS-exposure only is not sufficient to describe procoagulant platelets as platelets can have PS-exposure while not being procoagulant. While this study has confirmed the formation of two populations in platelet response with regards to Annexin V and PAC-1 binding and the intrinsic character of this heterogeneity other aspects of procoagulant platelets were not investigated. The PS-exposing population was shown to be present in response to both convulxin only and dual agonist stimulation but was bigger in response to dual agonist stimulation. This is also described in literature.[45, 49] This study also found that P-selectin and CD63 expression are associated with platelet activation and not related to the heterogeneity of procoagulant platelets. P-selectin has been used by some studies as an activation label independent of procoagulant features.[54] Procoagulant platelets have been described as ballooning and microvesiculating.[60] However, this study found a decrease of forward scatter and, for single platelets, sideward scatter. These findings would indicate that the formation of microvesicles is more likely than the ballooning. This could possibly be because of a specific time point or because of the lack of stirring, although this remains unknown at the moment.

The most important limitation of this study is that it is small scale with only two donors. However, the main aim of this study was to confirm findings already described in literature and find out if these were because of intrinsic variability of platelets. Because the collective platelets gave results similar to the literature it makes it more credible. The single platelet response however while showing that the heterogeneity is (mostly) intrinsic, needs repeating to explore the differences of platelet heterogeneity in the presence and absence of paracrine signalling. Furthermore, the research of hypersensitive subpopulations of platelets indicates that it is likely that the droplets are too small for single platelet function studies leading to problems controlling the concentrations of especially relatively large and very potent molecules like convulxin (~84 kDa) and thrombin (~72 kDa) and exaggeration of autocrine signalling caused by the small confined space. However, the effect of the droplet size on the heterogeneity has not been studied yet. Another limiting factor is the use of platelet rich plasma rather than washed platelet concentrates. This choice means it is harder to compare with literature and requires the use of inhibiting molecules such as Rivaroxaban and GPRP. Nevertheless, washed platelet concentrates require laborious handling of the platelets and to prevent activation during this process other inhibitors (such as apyrase) are necessary.[169] This method is not incompatible with washed platelets so the single platelet results should be compared with washed platelet in droplets in the future to study the effects of the different approaches. Finally, this study only used PAC-1 and

Annexin V to identify the procoagulant platelets. While PS-exposure is always present on procoagulant platelets, not all PS-exposing platelets are in fact procoagulant. Repeating some single platelet experiments with a target specific for procoagulant platelets such as labelled factor V could confirm if the PS-exposing single platelets are also procoagulant.

For further research the live imaging of platelet activation in droplets could be considered. This decreases the throughput but can provide more information on the single platelets especially with regards to changes in morphology. Furthermore, the confined compartments provide unique possibilities with regards to the investigation of effect that platelets have on fibrin formation. In addition, the restrictions on platelet encapsulation dictated by Poisson statistics means every assay contains 3 groups: fibrin formation without platelets, and fibrin formation with procoagulant platelets or proaggregatory platelets. Addition of labelled fibrinogen and a procoagulant label such as labelled factor V could provide new insights into the functionality of procoagulant platelets on thrombus formation. Encapsulation of multiple platelets in bigger droplets with coagulation can provide information on the reproducible formation of thrombi from heterogeneous and various platelets.

In conclusion, this study has confirmed that the heterogeneity of platelets with regards to their procoagulant phenotype is mostly intrinsic. Furthermore, that the formation of the procoagulant population is less susceptible to single platelet sensitivity loss than the activation of the $\alpha_{IIb}\beta_3$ receptor, and finally the procoagulant single platelets decrease in forward and sideward scatter while procoagulant collective platelets only decrease in forward scatter, probably by the formation of microvesicles.

Chapter 5 Discussion and conclusion

Platelet function is important for haemostasis and thrombosis. New insights into other cell types, such as T-cells, have shown that apparently homogeneous populations of cells have heterogeneous properties.[109] Furthermore, functional heterogeneity of platelets has been a topic of interest since the description of COAT platelets by Alberio et al.[45] While spatiotemporal influences have a big effect on platelets, intrinsic heterogeneity has been suggested before.[33, 58] However, because of the importance of the amplification of platelet response, by recruitment and activation of other platelets in the vicinity, intrinsic variability cannot be studied using conventional platelet function methods. To solve this problem, this study developed a method that combines droplets microfluidics with flow cytometry for the high throughput analysis of single platelet function and intrinsic variability of platelets. Understanding the intrinsic variability of platelets can improve the knowledge on thrombus formation that is important for the development of diagnosis and treatments for dysfunctional platelets and abnormal thrombus formation.

This study developed a new assay for the investigation of single platelets. This assay combines droplet microfluidics and flow cytometry for the high throughput analysis of single platelet response to stimuli. This assay is novel because of the combination of methods and the application of droplet microfluidics to a new cell type rather than its individual components. Every step in the protocol was optimised to improve the signal to noise and enable the reliable and reproducible study of single platelets. The final assay consists of extracting a platelet suspension from freshly donated blood, dilution of the platelet suspension in buffer to ensure single platelet encapsulation following a Poisson distribution, encapsulation of platelets in droplets in a solution of agonist and antibody, incubation within the droplets for 10-15 min and subsequent breaking of the emulsion in the presence of fixative and multiplexed flow cytometric analysis as the final end-point. Applying this new assay revealed a more than 10-fold sensitivity difference between single and collective platelets to convulxin. Furthermore, stimulation with other agonists also led to sensitivity differences between singularly and collectively stimulated platelets. Moreover, platelet sensitivity varied between single platelets from the same donor causing a continuous range of sensitivities. This has never been described before due to the lack of a high throughput method for the analysis of single platelets in confinement. It was also shown that the procoagulant platelets have a mostly intrinsic origin as suggested before. Furthermore, the procoagulant capacity of platelets seems to be less susceptible to the sensitivity differences between single and collective platelets.

The novel assay developed by this study has revealed new insights into differences between individual platelets. However, it has some limitations as well. This method is laborious and requires experienced operators which presents a first entry barrier for dissemination and wider scale application. Furthermore, this limits the number of conditions measured in each experiment and making triplicate measurements impractical. Using a device with bigger channels and producing bigger droplets can speed up the transitions of conditions but the maximum of conditions that can be tested within the 4 hour window after blood draw recommended for platelet function tests is still limited to 10 or 12. However, bigger droplets are required to solve the enhancement of autocrine signalling because of confinement of the platelet and its autocrine factors. This will increase the costs of the assay by using more reagents though. Another factor is the need for fixative in this assay. Fixative is required for the retrieval of platelets from droplets without the

possibility to react to paracrine signalling or the emulsion breaking procedure and to stop the reaction. However, fixative itself has a small effect on platelet function measured with flow cytometry. Moreover, the necessity of fixative limits the selection of labels being used because the labels need to be encapsulated with the platelets in the droplets, so only labels that do not elicit platelet activation can be used or need to be able to stain pre-fixed platelets. The six labels used in this study are suitable when diluted in buffer and encapsulated in droplets but not all labels will be suitable. In addition, the use of hydrophobic dyes such as membrane or mitochondrial dyes are not applicable because of they readily partition into the carrier fluoro-oil phase. The platelet suspension was diluted with buffer rather than autologous plasma or washed platelet concentrates to avoid disrupting the balance between pro- and anticoagulant factors and avoid the necessity of using platelet inhibitors such as apyrase. However, this confounds direct comparison to results from the literature.

While the current assay works and has provided new insights into platelet function a few improvements can be made. Increasing the droplet size can decrease the effects of autocrine signalling and speed up the stabilisation of flows. However, this requires extra dilution and has to be optimised further to find a balance between decreased autocrine signalling effects and increased dilution effects and costs. Furthermore, the development of a fully integrated microfluidic work flow, where droplet formation, incubation and breaking are all included, allows effective assay automation and can improve the time control over the incubation period. Additionally, imaging of the droplets on chip can increase the knowledge extracted per experiment. Studying thrombus formation in droplets, where more than one platelet is encapsulated, with or without coagulation can readily be undertaken.

To conclude, this study has developed a reliable, reproducible, high throughput method to test single platelet function and intrinsic variability of platelets within individuals. Using this method, a new variability in sensitivity levels has been discovered and an overall decreased sensitivity of single platelets. Furthermore, procoagulant capacity of platelets has been shown to be mostly intrinsic and less susceptible to single platelet sensitivity loss. These new insights can lead to a better understanding of thrombus formation and how single platelet variety contributes to this. Ultimately, finding new drugs that specifically target hypersensitive platelets or screening existing drugs to find optimal (combinations of) dosages to target mostly hypersensitive platelets can improve existing treatment. The identification of patients at risk for thrombosis might be improved by the screening of the hypersensitive platelets. Therefore, this assay can improve our current understanding of platelet function and thrombus formation which in turn can lead to improved outlook for patients.

Appendix A

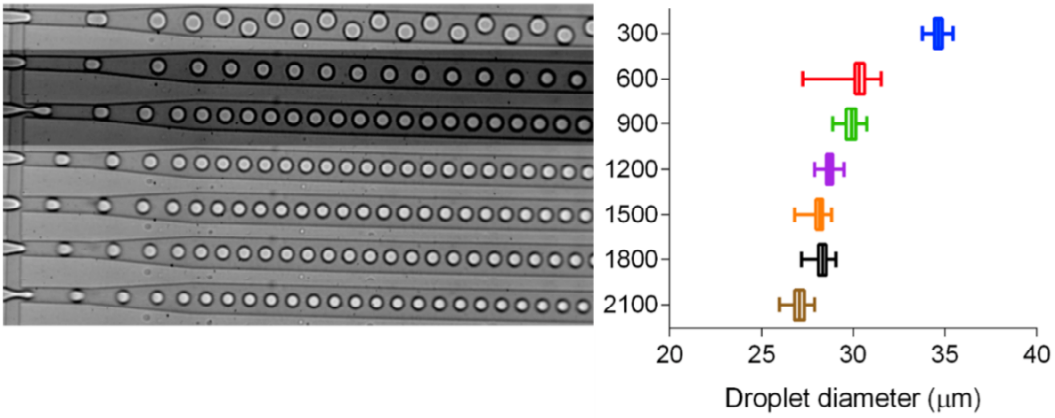


Figure A-1 The effect of changing the total flow rate on the size of the droplets. The total flow rate of droplet formation was varied between 300 and 2100 μL/hr while keeping the oil to aqueous ratio equal at 1:4. **A)** representative images of live droplet formation, **B)** box and whisker plot of droplet sizes, whiskers represent 10th and 90th percentile. Droplet surface determined with image analysis of live droplet videos using ImageJ. These results are discussed in section 2.3.2

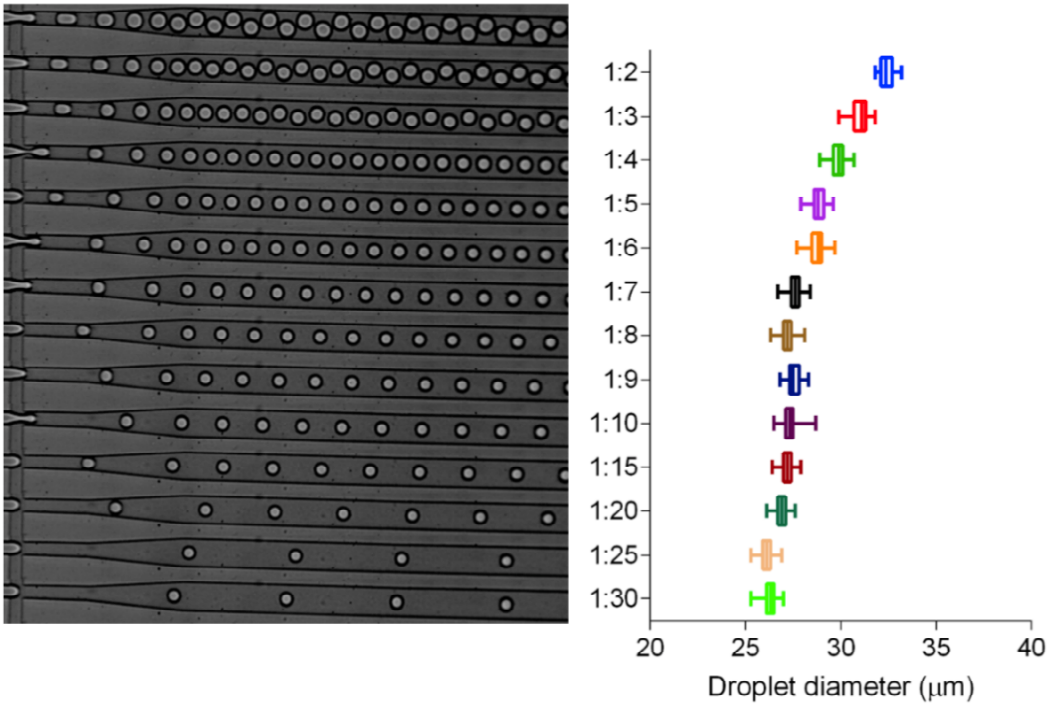


Figure A-2 The effect of changing the flow ratio of oil to aqueous input on the size of the droplets. The oil to aqueous ratio of droplet formation was varied between 2:1 and 30:1 while keeping the total flow rate equal at 900 μL/hr. **A)** representative images of live droplet formation, **B)** box and whisker plot of droplet sizes, whiskers represent 10th and 90th percentile. Droplet surface determined with image analysis of live droplet videos using ImageJ. These results are discussed in section 2.3.2.

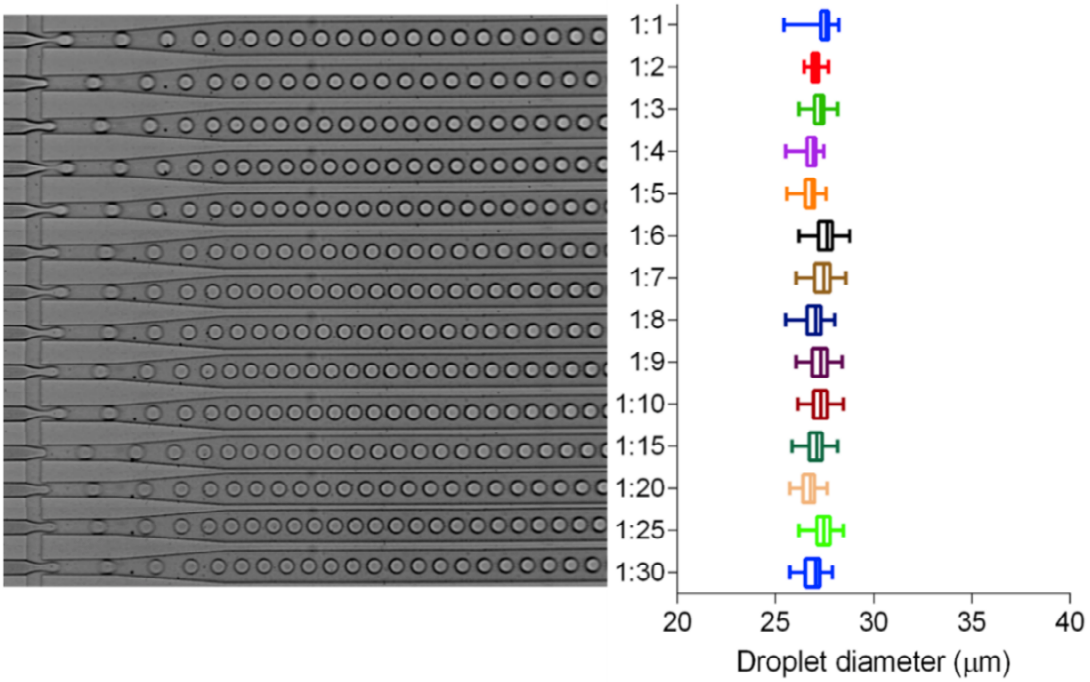


Figure A-3 The effect of changing the flow ratio of the aqueous inlets on the size of the droplets. The ratio was varied between 1:1 and 1:30 while keeping the total flow rate equal at 900 μL/hr and the oil to water ratio at 4:1. **A)** representative images of live droplet formation, **B)** box and whisker plot of droplet sizes, whiskers represent 10th and 90th percentile. Droplet surface determined with image analysis of live droplet videos using ImageJ. These results are discussed in section 2.3.2.

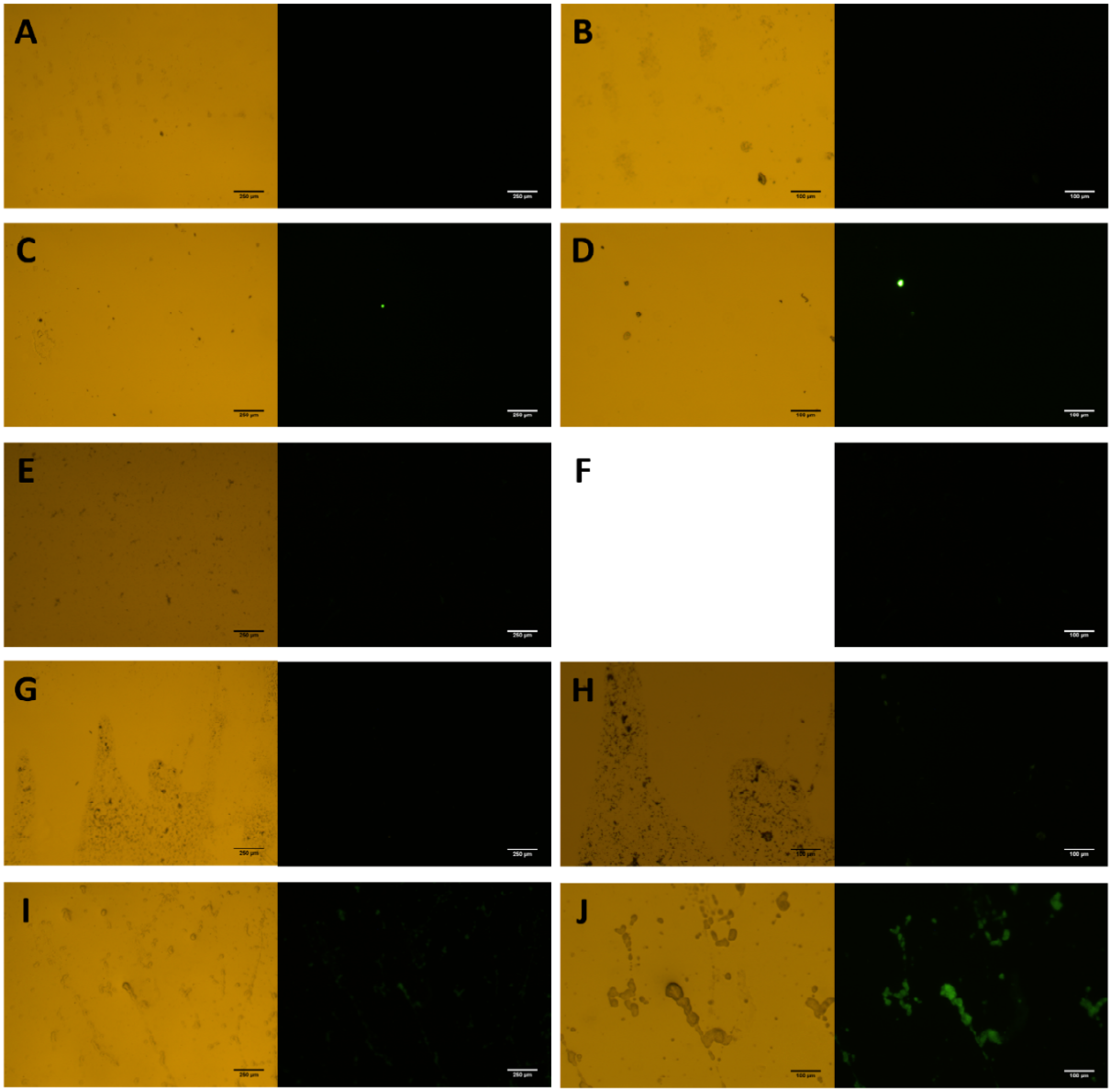


Figure A-4 Effect of surface treatments of PDMS on platelet adhesion. **A** and **B)** PDMS blocked with 1% BSA. **C** and **D)** PDMS treated with oxygen plasma and subsequently blocked with 1% BSA. **E** and **F)** PDMS treated with oxygen plasma and subsequently incubated overnight at 60 °C. **G** and **H)** PDMS treated with oxygen plasma. **I** and **J)** PDMS spot-coated with collagen and subsequently blocked with 1% BSA. **A, C, E, G, I)** microscopy image of brightfield and FITC with 4x magnification, scale bar depicts 250 µm. **B, D, F, H, J)** brightfield and FITC channel zoomed in on same region with 10x magnification, scale bar depicts 100 µm. Platelet rich plasma stained with $DiOC_6$. These results are discussed in section 2.3.4.

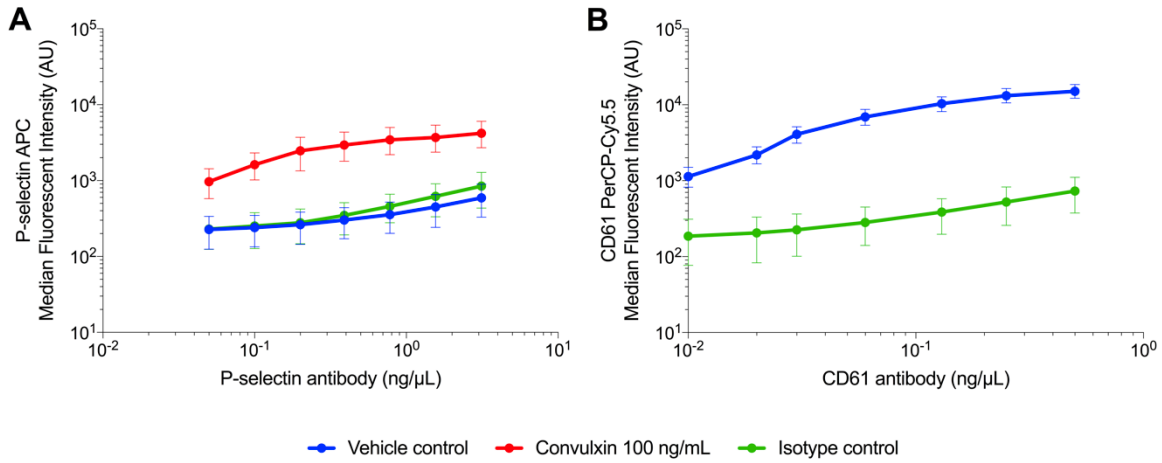


Figure A-5 Median fluorescence intensity of antibody titration of flow cytometry samples activated with convulxin (100 ng/mL) compared to the vehicle (not activated) and isotype control. **A)** P-selectin antibody and **B)** CD61 antibody. The MFI is obtained from the forward and sideward scatter gate, with median intensity plotted with quartiles. Used concentrations for anti-P-selectin were: 0.05, 0.10, 0.20, 0.39, 0.78, 1.56 and 3.13 ng/µL and a concentration of 0.39 ng/µL was chosen for further experiments. For anti-CD61 concentrations used were: 0.01, 0.02, 0.03, 0.06, 0.13, 0.25, 0.50 ng/µL and a concentration of 0.06 ng/µL was chosen for further experiments. These results are discussed in section 2.3.6.

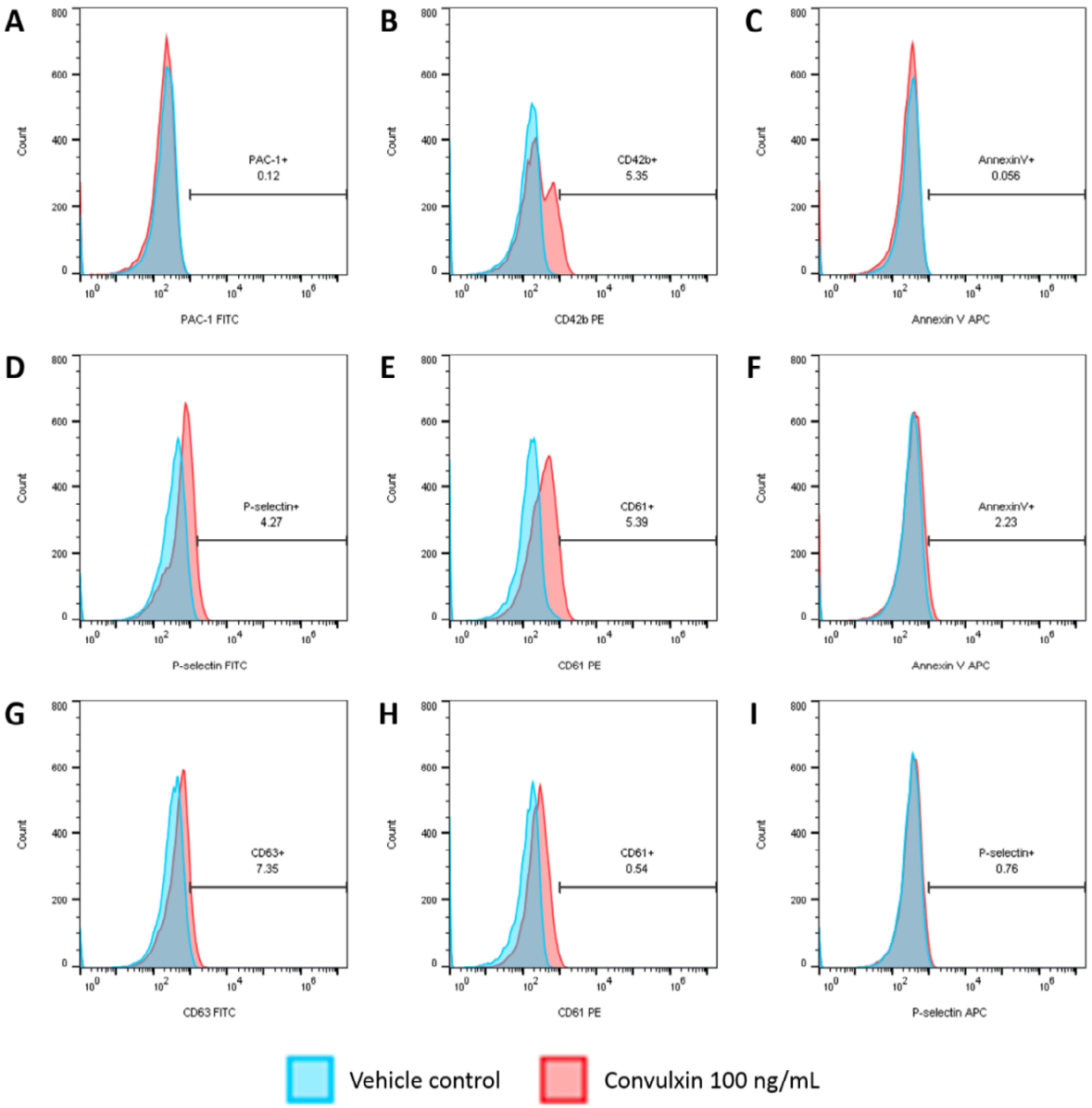


Figure A-6 A fluorescence minus one control for the simultaneous use of **A, B, C)** FITC conjugated PAC-1, PE conjugated anti-CD42b and APC conjugated Annexin V. **D, E, F)** FITC conjugated anti-P-selectin, PE conjugated anti-CD61 and APC conjugated Annexin V. **G, H, I)** FITC conjugated anti-CD63, PE conjugated anti-CD61 and APC conjugated anti-P-selectin. Graphs depict the signal in the channel of the fluorochrome left out. Events shown based on a forward and sideward scatter platelet gate. Comparing bleed through and background of platelets stimulated with vehicle in blue and 100 ng/mL convulxin in red. These results are discussed in section 2.3.7.

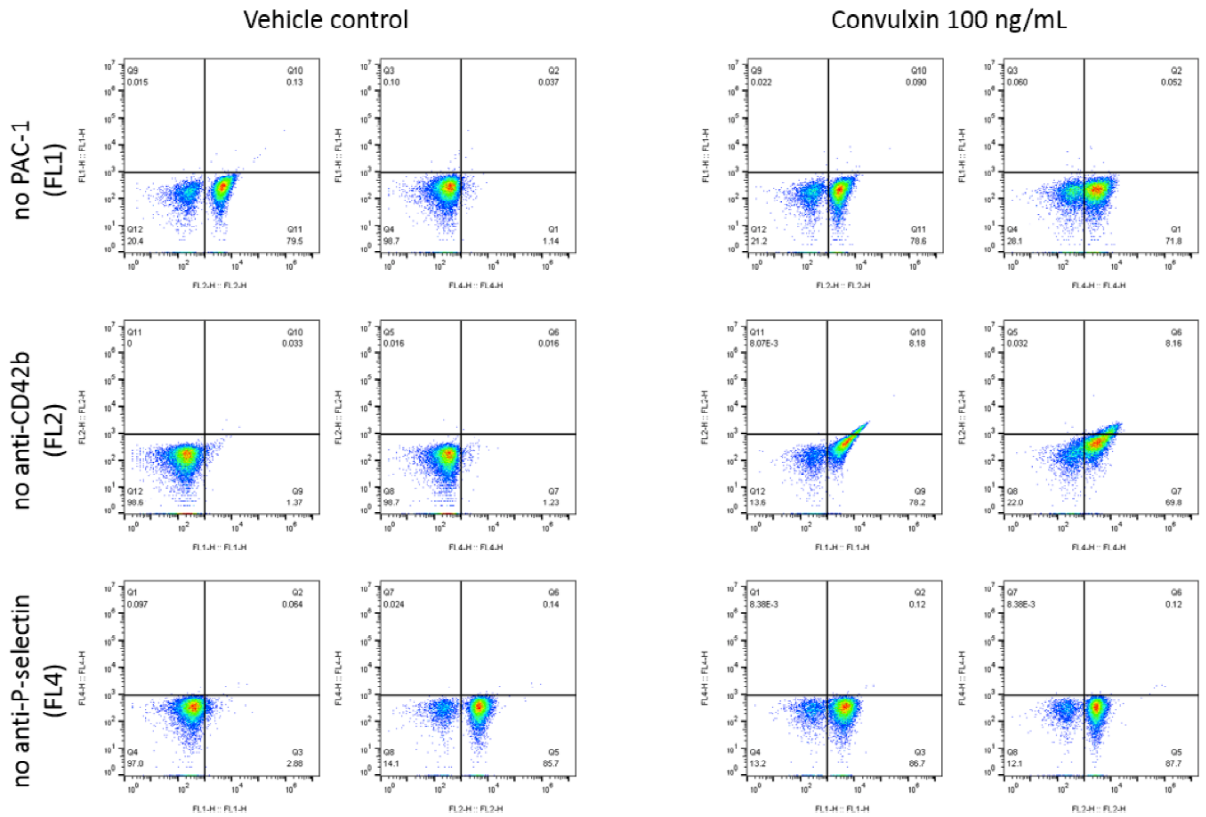


Figure A-7 Raw data scatter plots of the FMO control of Panel A. These results are discussed in section 2.3.7.

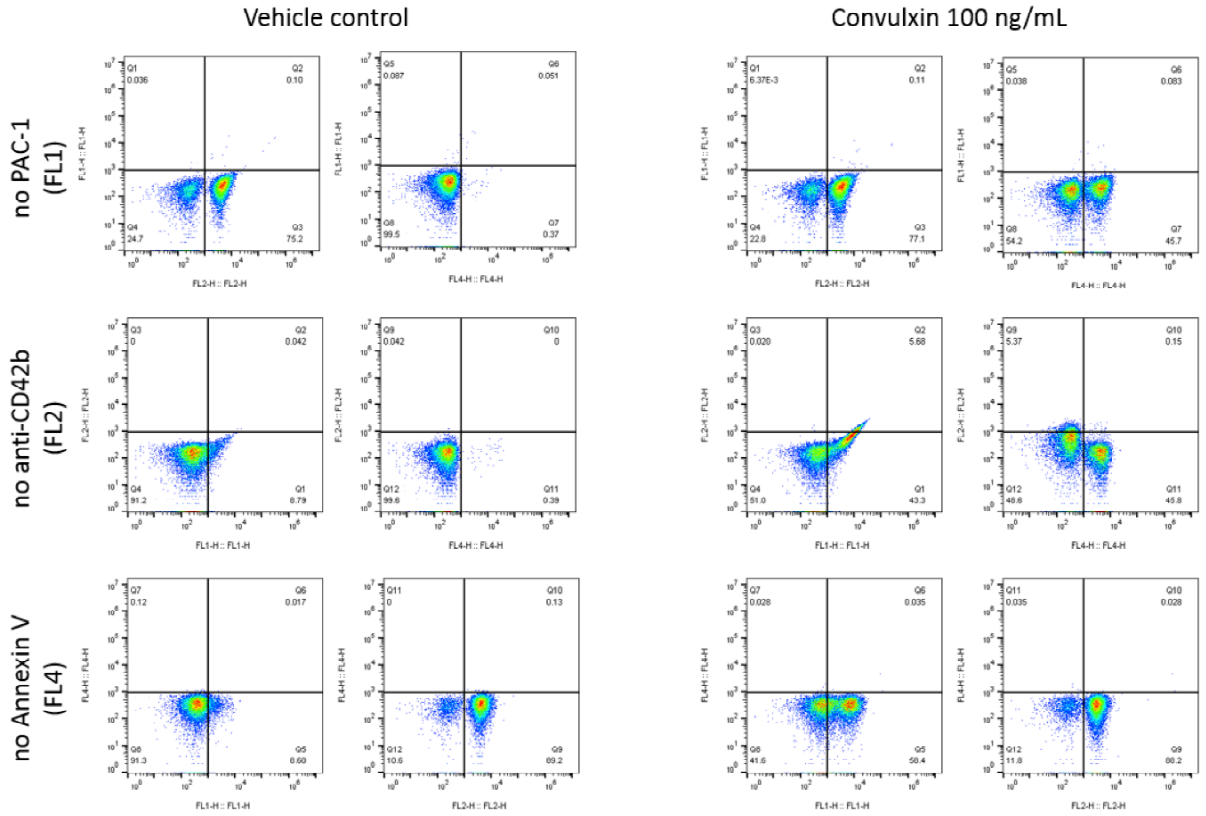


Figure A-8 Raw data scatter plots of the FMO control of Panel B. These results are discussed in section 2.3.7.

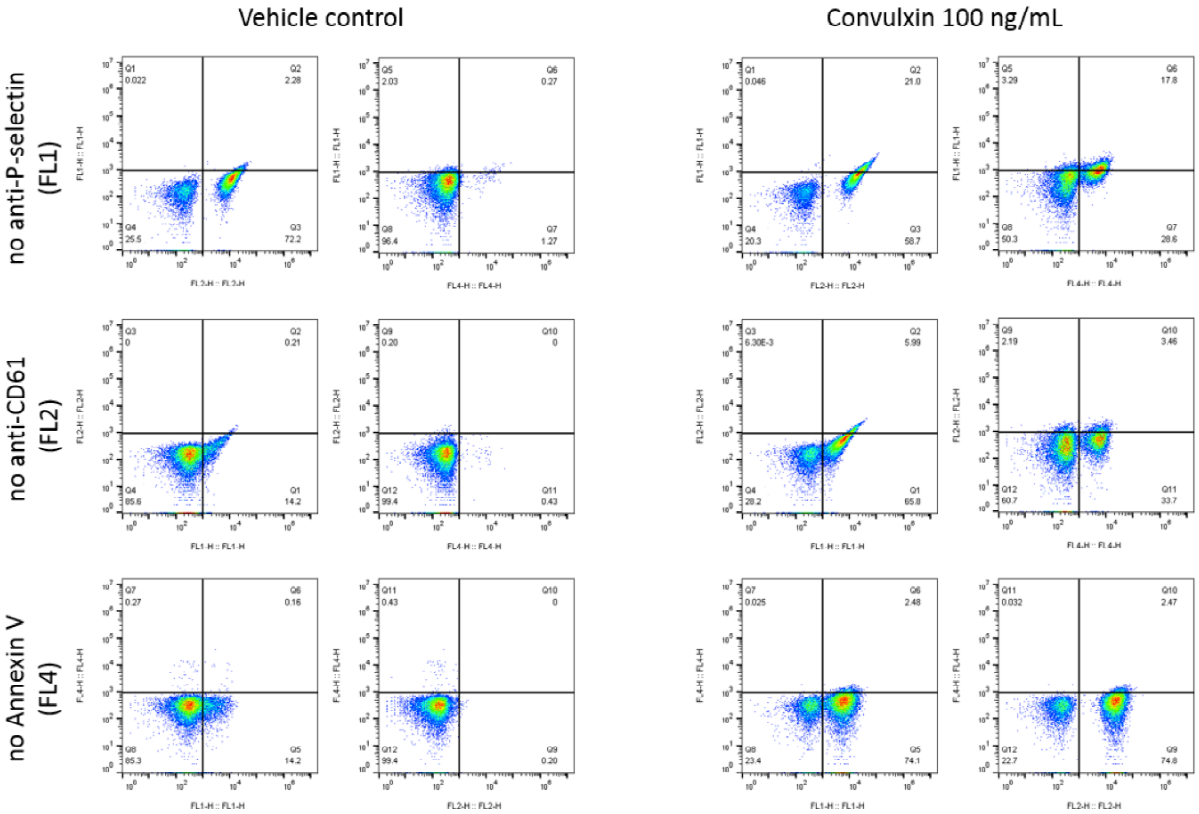


Figure A-9 Raw data scatter plots of the FMO control of Panel C. These results are discussed in section 2.3.7.

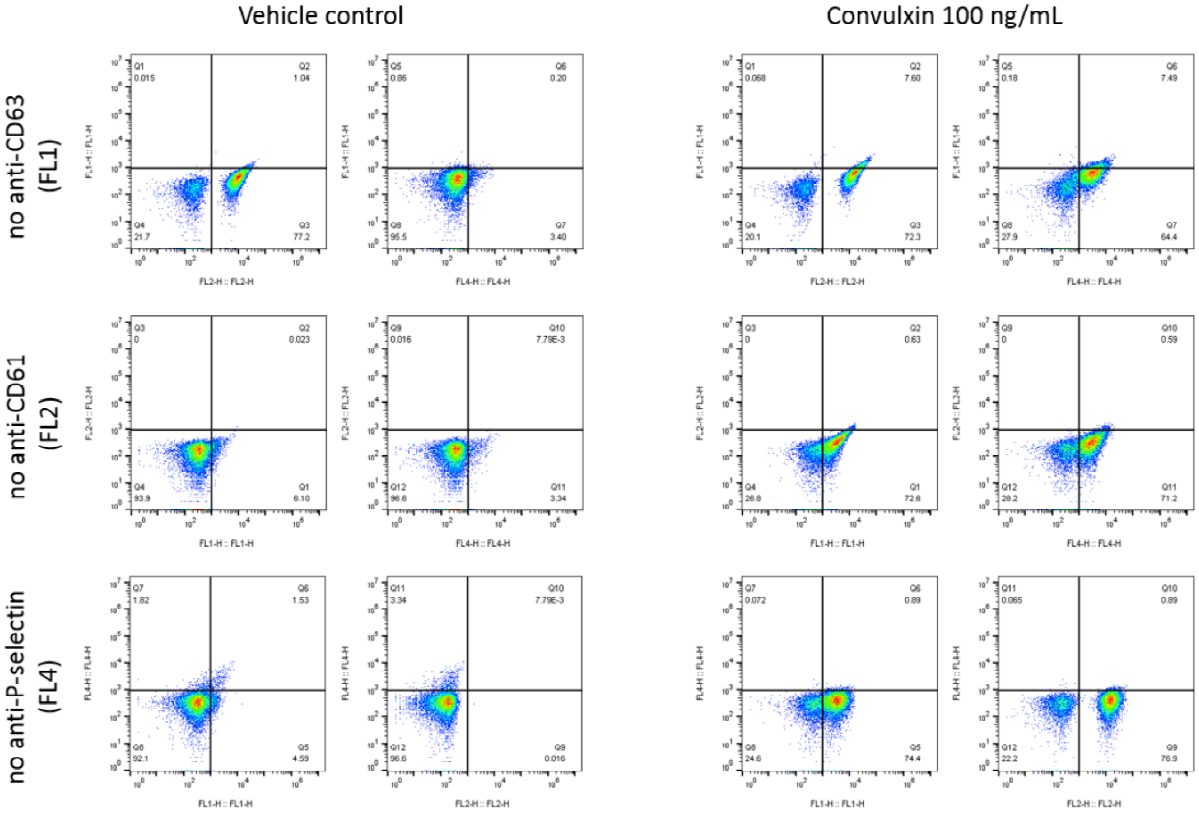


Figure A-10 Raw data scatter plots of the FMO control of Panel D. These results are discussed in section 2.3.7.

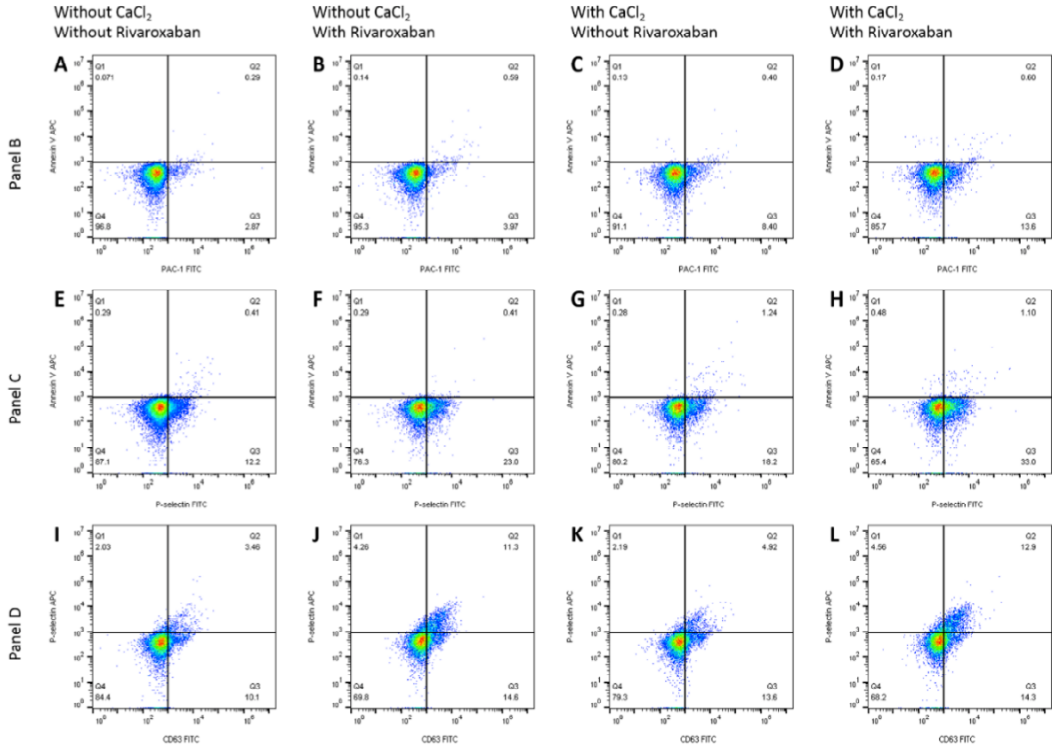


Figure A-11 The effect of Rivaroxaban and $CaCl_2$ on platelet activation. Platelets were incubated in buffer with or without Rivaroxaban for min 15 min. Subsequently, this platelet suspension was added to agonist and antibody solution with or without added $CaCl_2$. Platelet activity measured with **A-D)** PAC-1 and Annexin V (panel B), **E-H)** anti-P-selectin and Annexin V (panel C), **I-L)** anti-CD63 and anti-P-selectin (panel D). Platelet activity in the **A, E** and **I)** absence of $CaCl_2$ and Rivaroxaban. **B, F** and **J)** presence of Rivaroxaban and absence of $CaCl_2$. **C, G** and **K)** presence of $CaCl_2$ and absence of Rivaroxaban. **D, H** and **L)** presence of both $CaCl_2$ and Rivaroxaban. Platelets not activated (vehicle control).

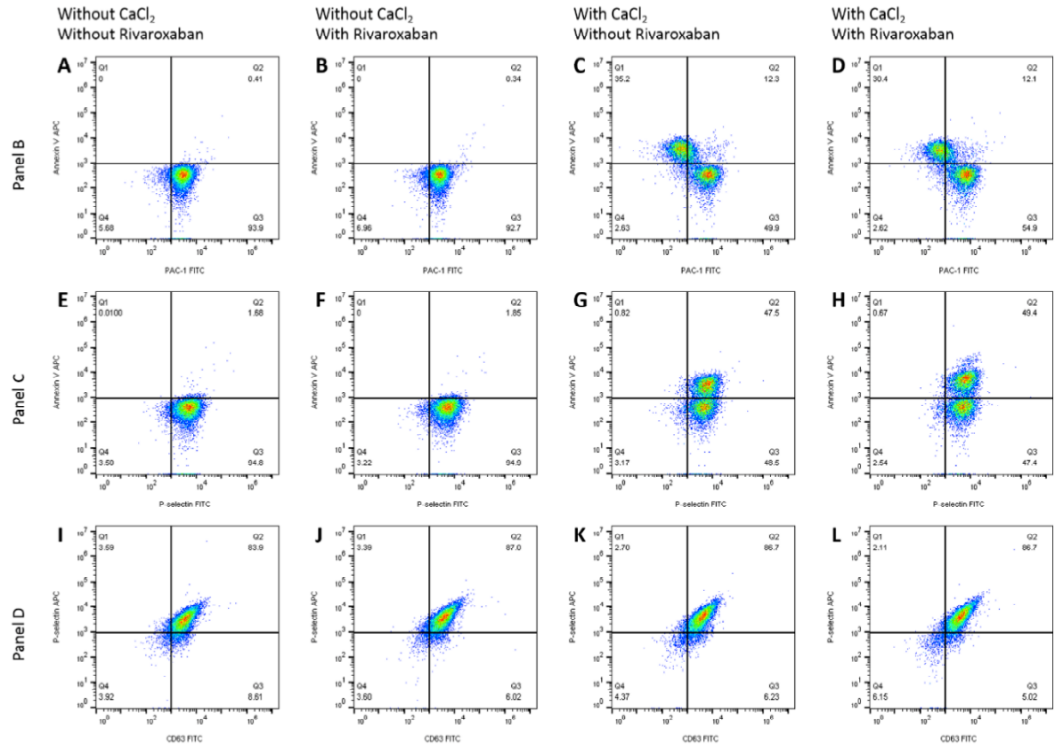


Figure A-12 The effect of Rivaroxaban and $CaCl_2$ on platelet activation. Platelets were incubated in buffer with or without Rivaroxaban for min 15 min. Subsequently, this platelet suspension was added to agonist and antibody solution with or without added $CaCl_2$. Platelet activity measured with **A-D)** PAC-1 and Annexin V (panel B), **E-H)** anti-P-selectin and Annexin V (panel C), **I-L)** anti-CD63 and anti-P-selectin (panel D). Platelet activity in the **A, E** and **I)** absence of $CaCl_2$ and Rivaroxaban. **B, F** and **J)** presence of Rivaroxaban and absence of $CaCl_2$. **C, G** and **K)** presence of $CaCl_2$ and absence of Rivaroxaban. **D, H** and **L)** presence of both $CaCl_2$ and Rivaroxaban. Platelets activated with 100 ng/mL of convulxin.

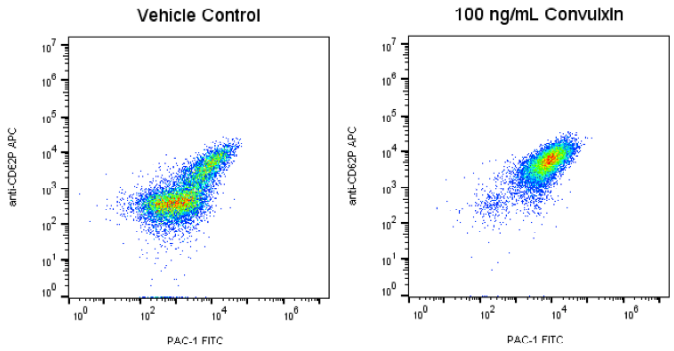


Figure A-13 Effect of adding antibodies directly to the PRP. Approximately 40% in the vehicle control is above the threshold and around 85% in the activated sample. These results were discussed in section 2.3.10.

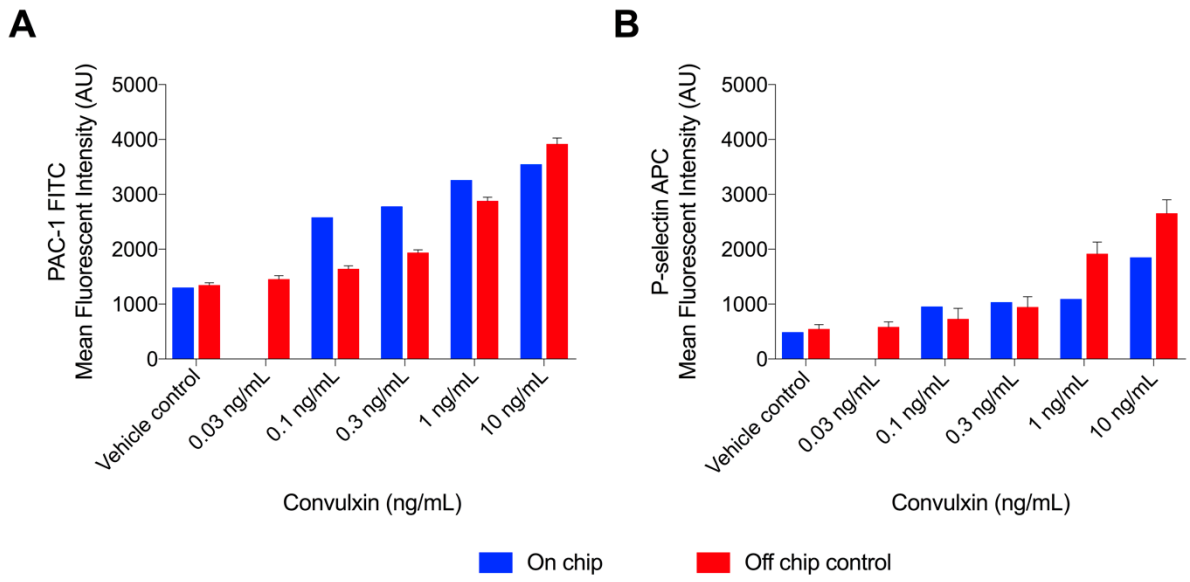


Figure A-14 Dose response relationship of platelet activity in response to convulxin. Platelet activity measured with **A)** PAC-1 antibody and **B)** P-selectin antibody. Platelets were stimulated on chip (in droplets, blue) or off chip (in suspension, red). These results were discussed in section 2.3.11.

Table A-1 Relative activation and confidence intervals of dose response relationship of single platelet activity (pre-incubated with antibodies and in droplets) in response to convulxin. Platelet activity measured with PAC-1 antibody and P-selectin antibody. This experiment was discussed in section 2.3.11.

Singular vehicle vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.1	4.9240	4.7314	5.1201	1.2424	1.2288	1.2547
0.3	4.4438	4.2562	4.6365	1.3019	1.2864	1.3166
1	6.7276	6.4536	7.0079	1.3932	1.3776	1.4077
10	8.1781	7.5436	8.8677	2.6162	2.5403	2.6951

Table A-2 Relative activation and confidence intervals of on chip comparison antibody binding and isotype control. Platelet activity in response to 100 ng/mL convulxin and vehicle control measured with PAC-1 antibody and P-selectin antibody and their respective isotype controls. This experiment was discussed in section 2.3.11.

Singular antibody vs isotype control response						
	PAC-1			P-selectin		
Comparison	Relative activation	Confidence interval		Relative activation	Confidence interval	
Antibody vehicle vs antibody convulxin	15.1730	13.5250	17.0331	2.4137	2.3369	2.4947
Isotype vehicle vs antibody vehicle	1.2760	1.2619	1.2904	1.0221	1.0170	1.0265
Isotype convulxin vs antibody vehicle	1.2832	1.2700	1.2971	1.0276	1.0244	1.0312
Isotype vehicle vs isotype convulxin	0.9943	0.9897	0.9975	0.9946	0.9906	0.9970
Isotype convulxin vs antibody convulxin	19.4700	17.3642	21.8473	2.4803	2.4018	2.5631

Table A-3 Relative activation and confidence intervals of different methods of breaking the emulsion (droplets). Platelet activity in response to 100 ng/mL convulxin and vehicle control measured with PAC-1 antibody and P-selectin antibody. Different breaking methods were: indirect (droplets collected and incubated in a tube before adding fixative and PFO), indirect with mineral oil (droplets collected underneath a layer of mineral oil and incubated in a tube before adding fixative and PFO) or direct (droplets incubated in long tubing and collected directly in tube with fixative and PFO). Compared with off chip control (same platelet suspension with antibodies added to agonist in a tube on the bench before fixation). This experiment was discussed in section 2.3.11.

Vehicle vs 100 ng/mL convulxin response						
	PAC-1			P-selectin		
Breaking method	Relative activation	Confidence interval		Relative activation	Confidence interval	
On chip Indirect	8.9214	7.2824	10.9363	10.0250	9.0964	11.0568
On chip Mineral oil	8.0985	6.5170	10.0683	12.8980	11.5646	14.3958
On chip Direct	2.1801	1.8729	2.5382	3.0409	2.8782	3.2152
Off chip	18.1340	16.8807	19.4849	7.1346	6.8777	7.4027

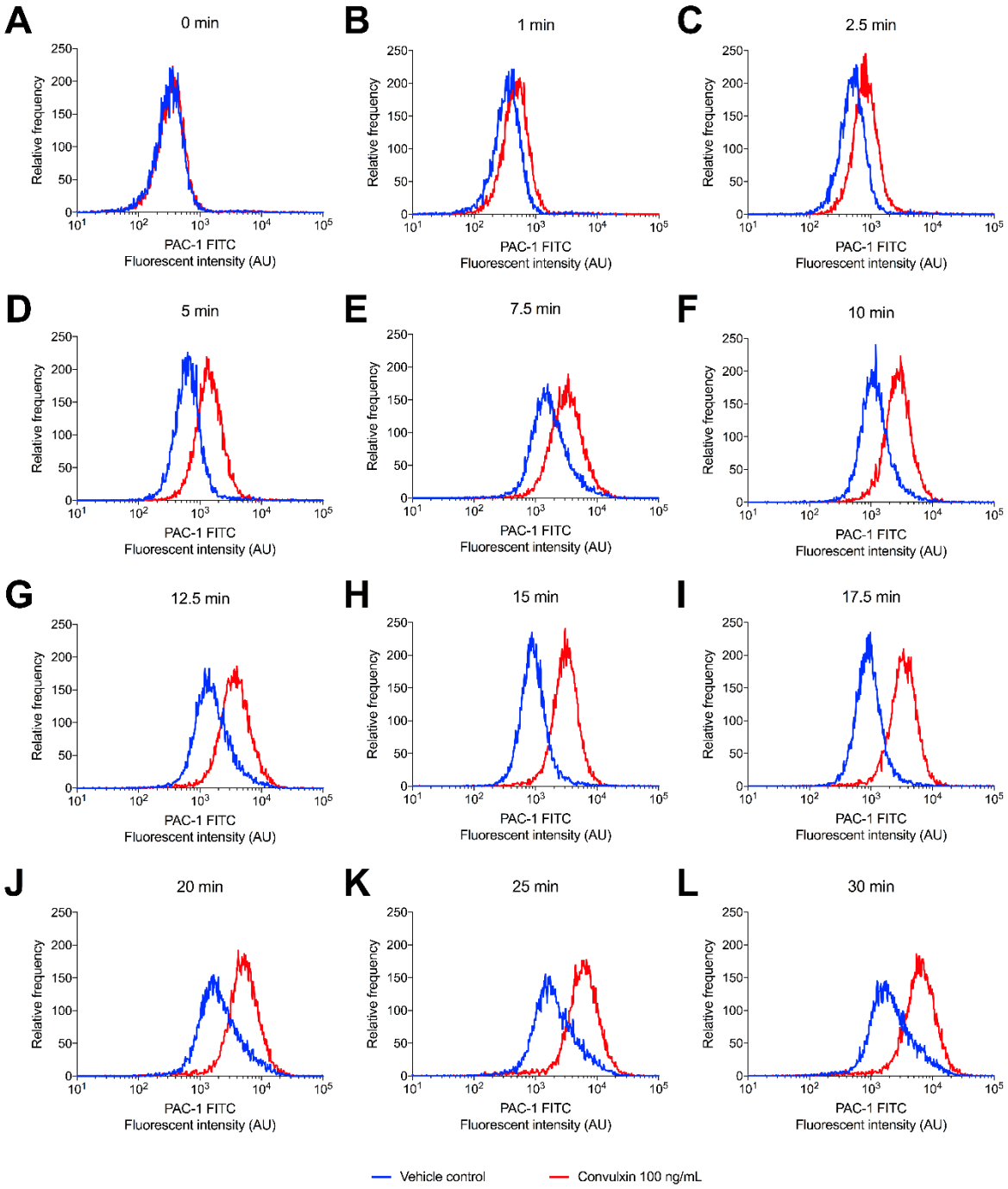


Figure A-15 Effect of incubation time of a pre-incubated with antibody platelet suspension in an agonist solution on signal to noise of stimulated and non-stimulated platelets. Platelet activity was measured with PAC-1 antibody and platelets were activated with 100 ng/mL of convulxin (red) compared to the vehicle control (blue).Platelets were incubated for **A)** 0 min, **B)** 1 min, **C)** 2.5 min, **D)** 5 min, **E)** 7.5 min **F)** 10 min, **G)** 12.5 min, **H)** 15 min, **I)** 17.5 min **J)** 20 min, **K)** 25 min and **L)** 30 min before addition of fixative to stop the reaction. This experiment was discussed in section 2.3.12.

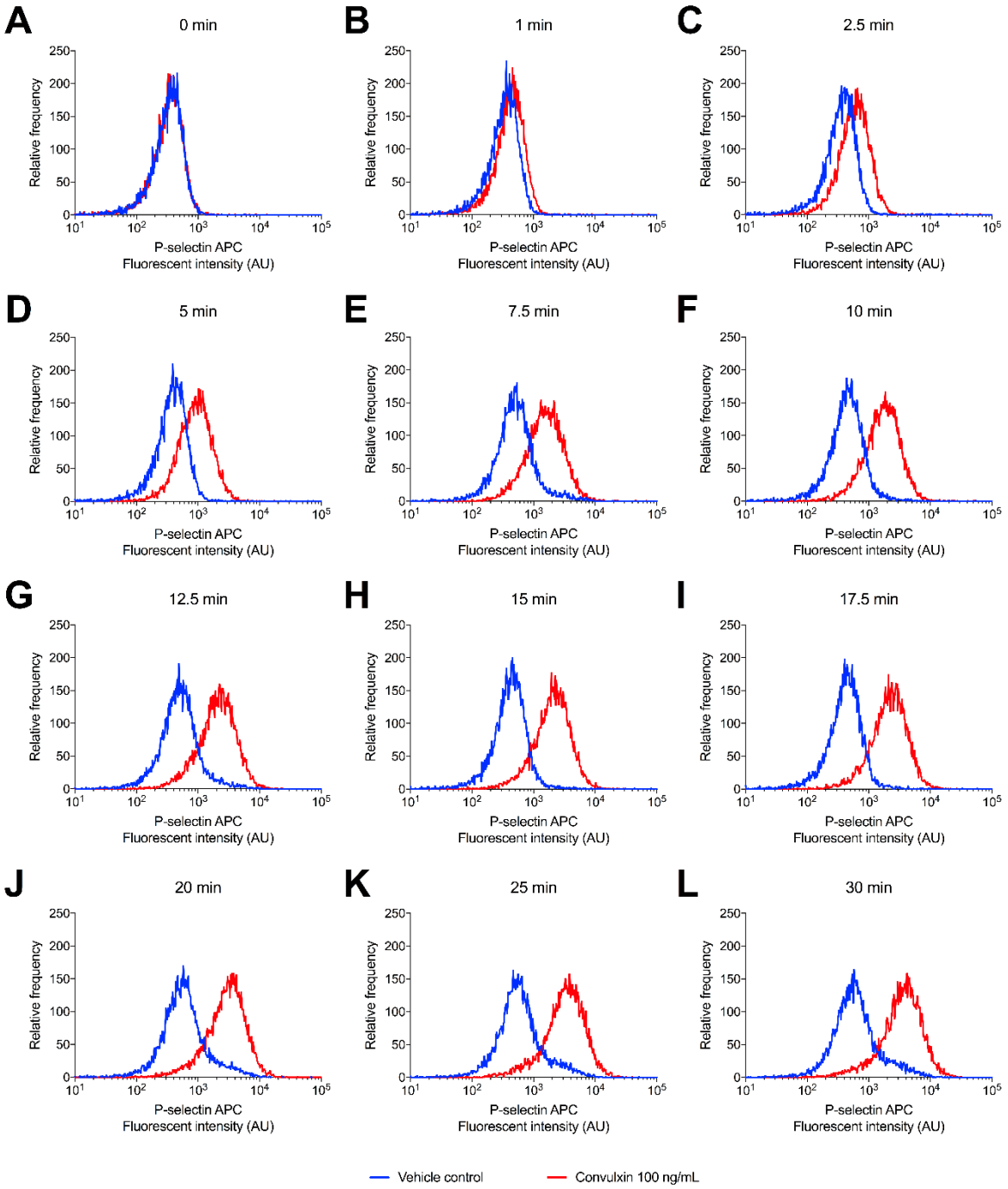


Figure A-16 Effect of incubation time of a pre-incubated with antibody platelet suspension in an agonist solution on signal to noise of stimulated and non-stimulated platelets. Platelet activity was measured with Pselectin antibody and platelets were activated with 100 ng/mL of convulxin (red) compared to the vehicle control (blue). Platelets were incubated for **A)** 0 min, **B)** 1 min, **C)** 2.5 min, **D)** 5 min, **E)** 7.5 min **F)** 10 min, **G)** 12.5 min, **H)** 15 min, **I)** 17.5 min **J)** 20 min, **K)** 25 min and **L)** 30 min before addition of fixative to stop the reaction. Platelet activity was measured with Pselectin antibody and platelets were activated with 100 ng/mL of convulxin compared to the vehicle control. This experiment was discussed in section 2.3.12.

Table A-4 Relative activation and confidence intervals of incubation time of a platelet suspension pre-incubated with antibody solution. Platelet activity in response to 100 ng/mL convulxin and vehicle control measured with PAC-1 antibody and P-selectin antibody. This experiment was discussed in section 2.3.12.

Vehicle vs 100 ng/mL convulxin response						
	PAC-1			P-selectin		
Incubation time (min)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0	1.0038	0.9999	1.0078	1.0026	1.0004	1.0049
1	1.0377	1.0321	1.0434	1.0210	1.0176	1.0248
2.5	1.3682	1.3491	1.3879	1.1756	1.1655	1.1863
5	3.6483	3.5188	3.7840	1.7582	1.7277	1.7900
7.5	5.8986	5.2797	6.5919	3.3374	3.2273	3.4525
10	10.2455	9.2718	11.3253	4.3521	4.1913	4.5207
12.5	8.7014	7.7027	9.8326	5.5038	5.2594	5.7615
15	23.3111	20.6187	26.3648	6.1800	5.9023	6.4732
17.5	23.6954	20.9542	26.8052	7.2025	6.8501	7.5758
20	4.8596	4.3079	5.4833	8.6423	8.1220	9.1993
25	4.2327	3.7936	4.7237	9.3255	8.7393	9.9547
30	4.2569	3.7938	4.7777	9.6646	9.0400	10.3362

Table A-5 Relative activation and confidence intervals of staining strategies of platelets in droplets. Platelets were added to an agonist (100 ng/mL convulxin or vehicle control) and antibody mixture in the droplets using a 4 inlet device. Platelets were pre-incubated with antibodies and added to an agonist in the droplets using a 3 inlet device. This experiment is discussed in section 2.3.13.

Vehicle vs 100 ng/mL convulxin response						
	PAC-1			P-selectin		
Device	Relative activation	Confidence interval		Relative activation	Confidence interval	
4 inlets	39.5138	36.4862	42.7990	14.6371	13.9479	15.3625
3 inlets	13.0552	11.7841	14.4661	10.5806	10.0863	11.1012

Table A-6 Relative activation and confidence intervals of platelet response reproducibility in the same donor. Platelets were stimulated with convulxin in droplets and measured using PAC-1 and P-selectin antibodies. Three samples of the same donor were taken over a period of 9 months. These results are described in section 2.3.14.

Singular first vs second repeat response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	1.0216	1.0136	1.0297	1.0080	0.9970	1.0192
0.1	1.0296	1.0242	1.0350	1.0510	1.0415	1.0606
1	1.1333	1.1088	1.1584	1.1019	1.0775	1.1270
100	2.0807	1.8953	2.2842	1.5248	1.4225	1.6345

Singular first vs third repeat response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9205	0.9148	0.9263	0.8692	0.8612	0.8772
0.1	1.0115	1.0069	1.0160	1.0407	1.0320	1.0494
1	0.8456	0.8336	0.8577	0.8404	0.8277	0.8532
100	1.8091	1.6395	1.9962	1.2925	1.2025	1.3892

Singular second vs third repeat response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9011	0.8963	0.9058	0.8622	0.8560	0.8685
0.1	0.9824	0.9778	0.9869	0.9902	0.9824	0.9980
1	0.7461	0.7325	0.7599	0.7626	0.7485	0.7770
100	0.8695	0.7961	0.9496	0.8476	0.7992	0.8990

Table A-7 Relative activation and confidence intervals of platelet response reproducibility in the same donor. Platelets were stimulated with convulxin in suspension and measured using PAC-1 and P-selectin antibodies. Three samples of the same donor were taken over a period of 9 months. These results are described in section 2.3.14.

Collective first vs second repeat response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9998	0.9988	1.0009	0.9899	0.9884	0.9914
0.1	0.9950	0.9811	1.0092	0.9896	0.9827	0.9966
1	0.7315	0.6509	0.8221	0.8175	0.7843	0.8521
100	0.5354	0.4772	0.6007	0.8197	0.7897	0.8507

Collective first vs third repeat response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	1.0050	1.0038	1.0062	1.0019	1.0001	1.0036
0.1	0.5886	0.5820	0.5952	0.8492	0.8443	0.8543
1	0.4175	0.3759	0.4638	0.5216	0.5025	0.5414
100	0.5911	0.5259	0.6642	0.9103	0.8764	0.9456

Collective second vs third repeat response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	1.0051	1.0039	1.0063	1.0121	1.0105	1.0136
0.1	0.5915	0.5849	0.5982	0.8582	0.8531	0.8632
1	0.5708	0.5191	0.6276	0.6380	0.6161	0.6607
100	1.1040	1.0001	1.2187	1.1106	1.0711	1.1516

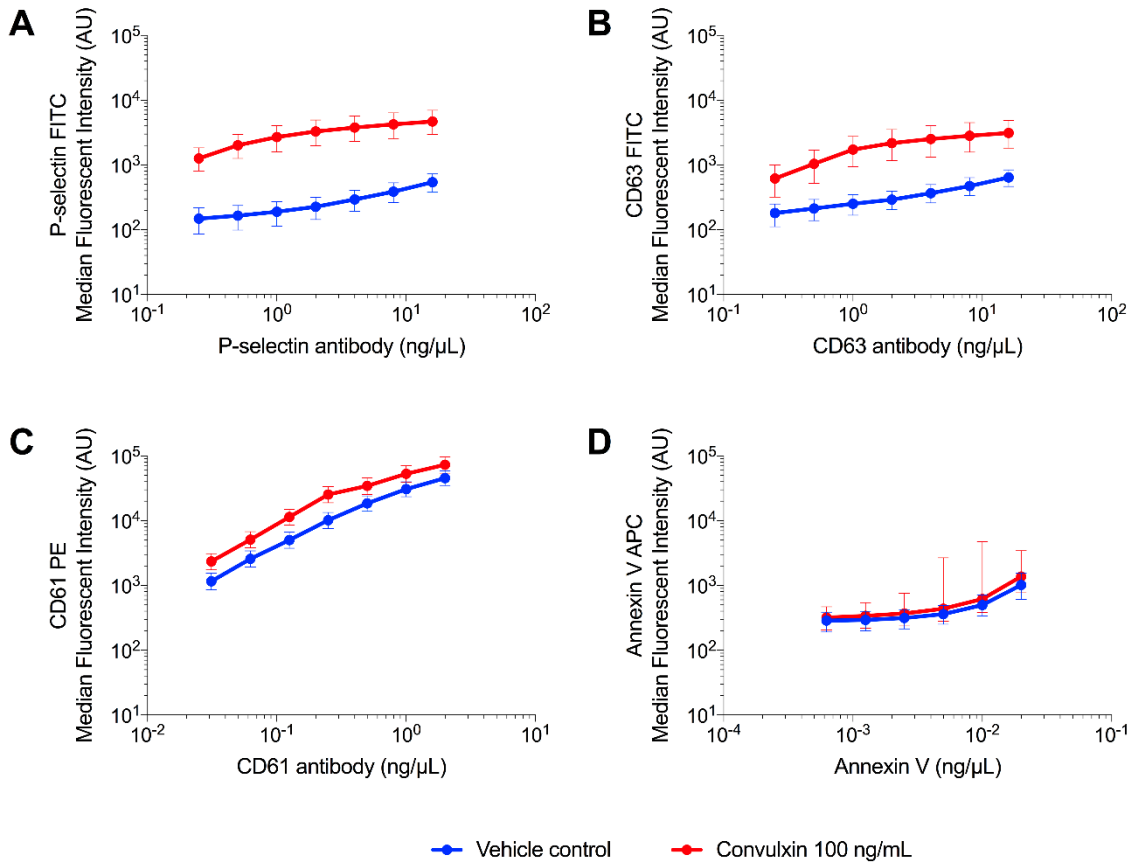


Figure A-17 Median fluorescence intensity of antibody titration of flow cytometry samples activated with convulxin (100 ng/mL) compared to the vehicle (not activated). **A)** P-selectin antibody, **B)** CD63 antibody, **C)** CD61 antibody and **D)** Annexin V. The MFI is obtained from the forward and sideward scatter gate, with median intensity plotted with quartiles. Used concentrations for anti-P-selectin were: 0.25, 0.5, 1, 2, 4, 8 and 16 ng/µL and a concentration of 2 ng/µL was chosen for further experiments. Used concentrations for anti-CD63 were: 0.25, 0.5, 1, 2, 4, 8 and 16 ng/µL and a concentration of 2 ng/µL was chosen for further experiments. For anti-CD61 concentrations used were: 0.03, 0.06, 0.13, 0.25, 0.5, 1 and 2 ng/µL and a concentration of 0.25 ng/µL was chosen for further experiments. Used concentrations for Annexin V were: 0.0006, 0.0013, 0.0025, 0.005, 0.01 and 0.02 ng/µL and a concentration of 0.08 ng/µL was chosen for further experiments after testing it separately.

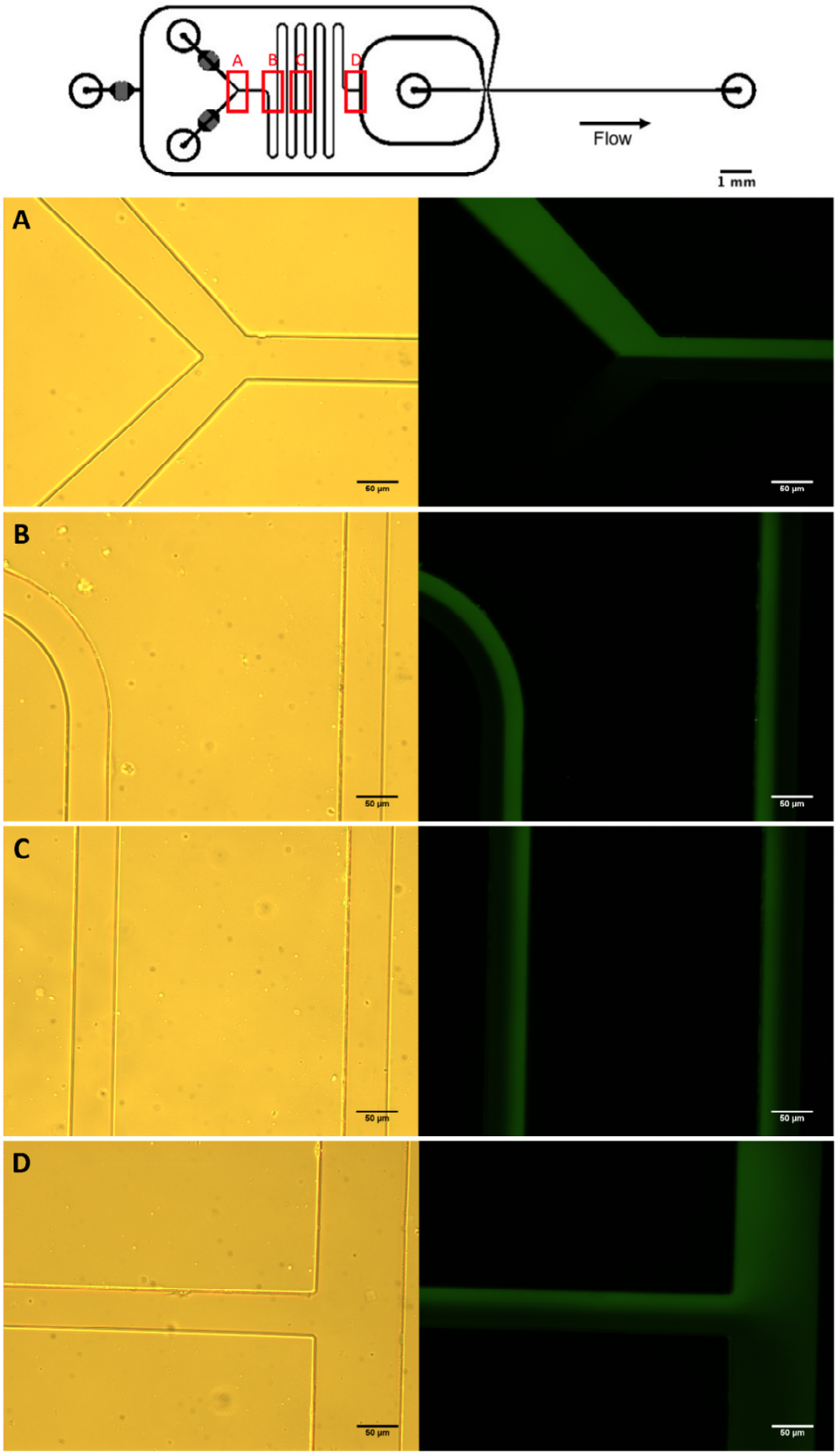


Figure A-18 Efficiency of the mixing structure of device shown in Figure 2-2. FITC labelled fluorescein is flowed through one of the 2 inlets used to mix agonist and antibody on chip and pictured at 4 sections within the device (legend shown above). Pictures kindly provided by Dr. Jonathan West.

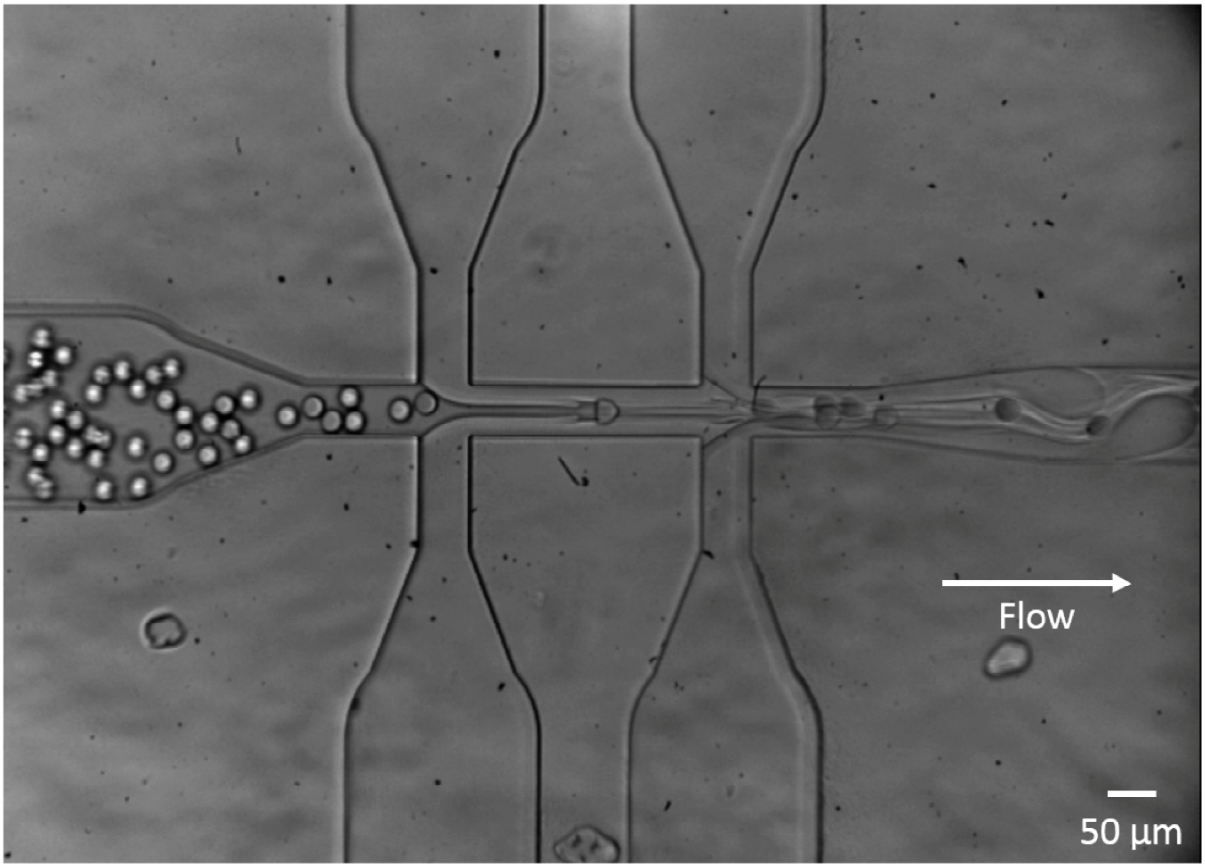


Figure A-19 Preliminary results of breaking the emulsion on chip. Droplets were mixed with PFO and subsequently with phosphate buffered saline (PBS).

Appendix B

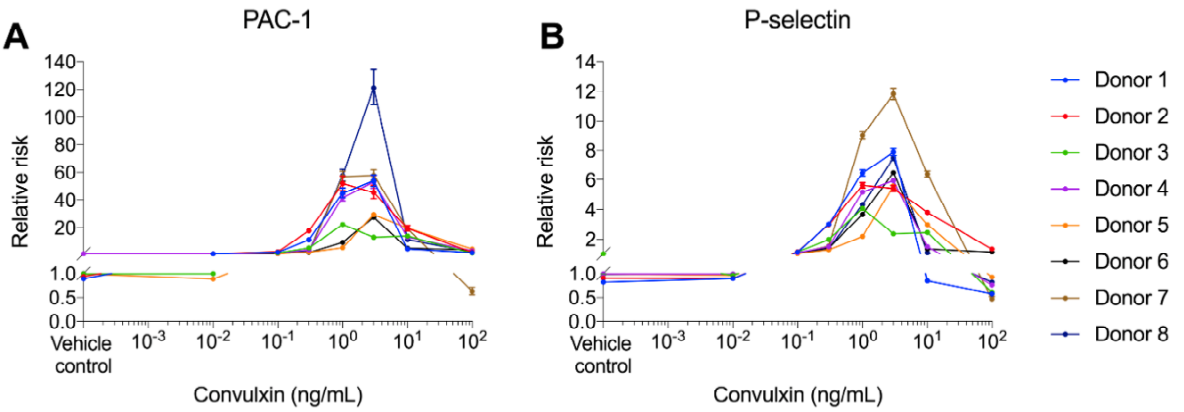


Figure B-1 The relative activation of a positive platelet response for collectively stimulated compared to singular stimulated platelets. Platelet response was measured with **A)** PAC-1 (integrin $\alpha_{IIb}\beta_3$ activation) and **B)** P-selectin (alpha granule secretion). Data is shown as relative activation with confidence interval. The confidence intervals were determined with the Koopman asymptotic score. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-1 GraphPad Prism output for the Emax model.

log(agonist) vs. response -- Variable slope (four parameters)	PAC-1		P-selectin	
	Singular	Collective	Singular	Collective
Best-fit values				
Bottom	419.2	396.1	412.7	363.6
Top	3857	4633	3224	2512
LogEC50	0.877	-0.3686	0.8753	-0.3374
HillSlope	2.047	1.736	2.208	2.224
EC50	7.533	0.428	7.505	0.4599
Span	3437	4237	2811	2148
Std. Error				
Bottom	77.58	149.7	70	50.6
Top	171.5	140.9	154.3	49.43
LogEC50	0.04869	0.06299	0.05196	0.04001
HillSlope	0.4106	0.3799	0.4969	0.3598
Span	191.4	215.8	171.5	72.79
95% CI (profile likelihood)				
Bottom	258.3 to 574.4	75.75 to 688.3	269.2 to 552.2	260.9 to 463.4
Top	3517 to 4212	4361 to 4922	2918 to 3538	2415 to 2610
LogEC50	0.775 to 0.975	-0.4966 to -0.2419	0.7646 to 0.9757	-0.4142 to -0.2563
HillSlope	1.3 to 3.505	1.141 to 2.942	1.367 to 5.005	1.644 to 3.194
EC50	5.956 to 9.44	0.3187 to 0.5729	5.815 to 9.456	0.3853 to 0.5542
Goodness of Fit				
Degrees of Freedom	60	76	60	76
R square	0.8808	0.8769	0.8581	0.9352
Adjusted R square	0.8749	0.8721	0.851	0.9326
Absolute Sum of Squares	13016193	40196443	10829115	5394130
Sy.x	465.8	727.3	424.8	266.4
Normality of Residuals				
Shapiro-Wilk W	0.8579	0.9518	0.8493	0.9215
P value	<0.0001	0.0044	<0.0001	0.0001
Passed normality test (alpha=0.05)?	No	No	No	No
P value summary	****	**	****	***
Replicates test for lack of fit				
SD replicates	476.8	755.7	437.9	275.6
SD lack of fit	268.3	190.4	152.3	113.1
Discrepancy (F)	0.3167	0.06349	0.121	0.1684
P value	0.8657	0.9989	0.9744	0.9844
Evidence of inadequate model?	No	No	No	No
Dependency				
Bottom	0.2266	0.3081	0.2134	0.2193
Top	0.3519	0.2973	0.3407	0.241
LogEC50	0.3642	0.3023	0.3616	0.2634
HillSlope	0.1975	0.2893	0.1905	0.2261
Number of points				
# of X values	80	80	80	80
# Y values analysed	64	80	64	80

Table B-2 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 1. This experiment was discussed in section 3.3.2. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.8882	0.8838	0.8928	0.8152	0.8097	0.8207
0.01	1.0215	1.0180	1.0251	0.8951	0.8885	0.9017
0.1	2.1223	2.0996	2.1452	1.1304	1.1215	1.1393
0.3	11.4425	11.0760	11.8212	3.0090	2.9605	3.0583
1	45.1419	41.7488	48.8108	6.4913	6.2815	6.7081
3	53.7012	49.6444	58.0895	7.9042	7.6699	8.1456
10	4.6260	4.1832	5.1158	0.8459	0.8021	0.8921
100	1.8402	1.6819	2.0133	0.5784	0.5521	0.6060

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	0.9031	0.8979	0.9083	0.9229	0.9138	0.9320
0.1	0.9349	0.9290	0.9408	0.9331	0.9247	0.9416
0.3	1.0258	1.0170	1.0346	0.9540	0.9446	0.9635
1	1.4061	1.3808	1.4319	1.2955	1.2713	1.3201
3	1.2267	1.2104	1.2433	1.0729	1.0589	1.0872
10	15.1505	14.2589	16.0979	8.5676	8.1737	8.9804
100	28.0121	26.4203	29.6998	12.0904	11.6181	12.5819

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0386	1.0364	1.0407	1.0133	1.0117	1.0149
0.1	2.2337	2.2113	2.2564	1.2938	1.2873	1.3004
0.3	13.2144	12.8007	13.6416	3.5213	3.4706	3.5728
1	71.4586	66.2179	77.1141	10.3157	10.0333	10.6059
3	74.1652	68.6300	80.1468	10.4031	10.1172	10.6970
10	78.9047	72.8049	85.5155	8.8902	8.6650	9.1213
100	58.0324	54.1909	62.1463	8.5787	8.3667	8.7961

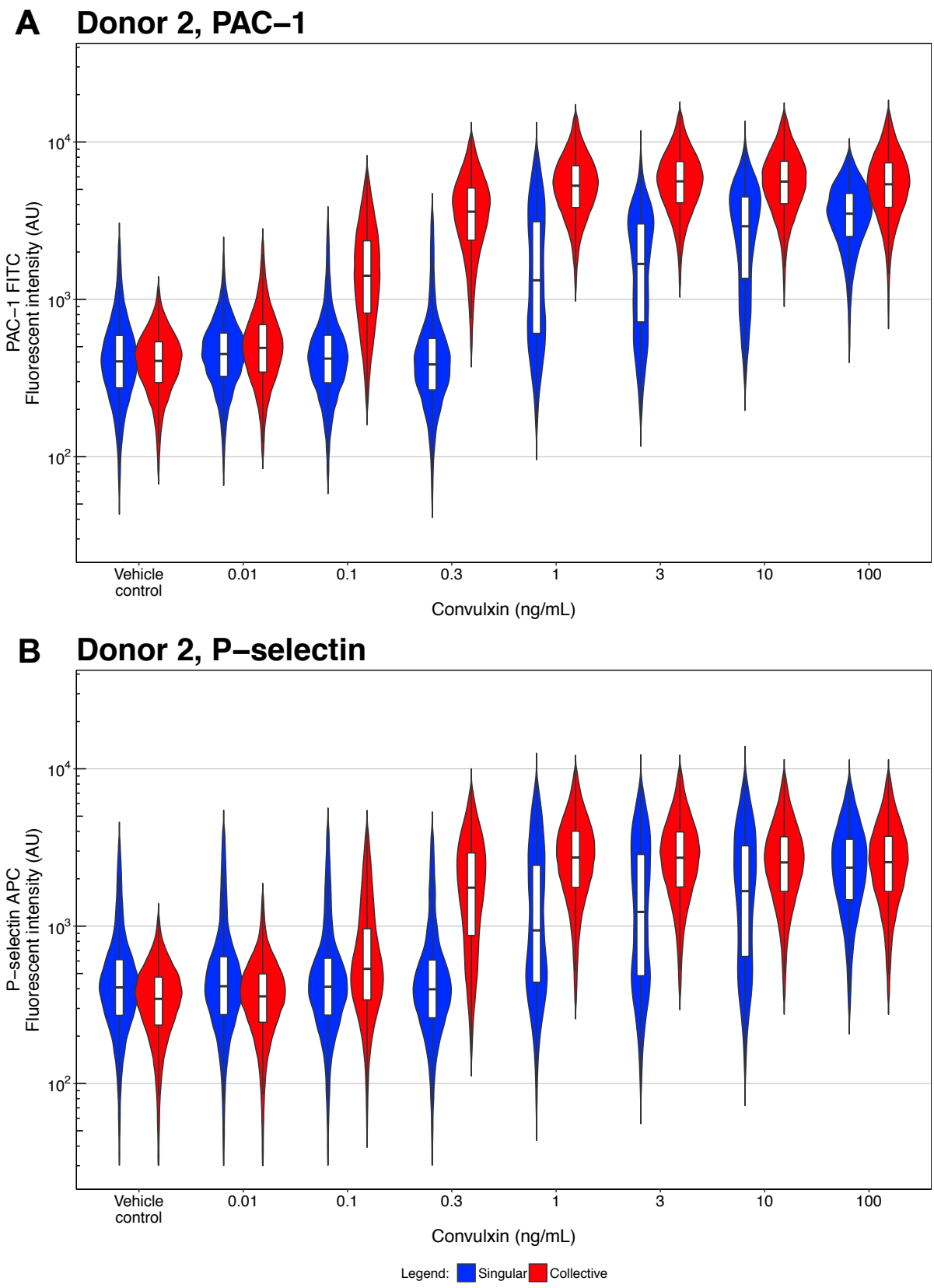


Figure B-2 Platelet responses of donor 2. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. This type of data is further discussed in section 3.3.2. N=8 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-3. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-3 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 2. This experiment was discussed in section 3.3.2. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9395	0.9351	0.9440	0.9022	0.8971	0.9073
0.01	1.0528	1.0473	1.0583	0.8990	0.8926	0.9054
0.1	2.7125	2.6763	2.7491	1.1476	1.1387	1.1566
0.3	17.9170	17.2228	18.6393	2.9870	2.9403	3.0344
1	51.7451	46.7941	57.2200	5.6381	5.4628	5.8191
3	45.5438	41.0350	50.5480	5.4479	5.2595	5.6431
10	19.4562	17.6007	21.5072	3.8198	3.6793	3.9657
100	2.3563	2.0726	2.6788	1.3514	1.2770	1.4301

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	0.9727	0.9666	0.9787	1.0196	1.0106	1.0287
0.1	1.0063	0.9998	1.0128	1.0234	1.0151	1.0317
0.3	1.0214	1.0135	1.0294	1.0161	1.0068	1.0254
1	2.1944	2.1557	2.2337	1.7204	1.6944	1.7467
3	2.6230	2.5617	2.6857	1.9617	1.9236	2.0005
10	5.0293	4.8338	5.2327	2.4785	2.4159	2.5428
100	28.2405	25.4654	31.3180	6.8896	6.5558	7.2404

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0899	1.0864	1.0935	1.0160	1.0140	1.0181
0.1	2.9052	2.8687	2.9423	1.3018	1.2950	1.3086
0.3	19.4788	18.7332	20.2541	3.3642	3.3173	3.4117
1	120.8577	109.4544	133.4489	10.7517	10.4520	11.0599
3	127.1498	114.8608	140.7536	11.8460	11.4982	12.2043
10	104.1502	94.9797	114.2062	10.4943	10.2049	10.7918
100	70.8264	65.6429	76.4192	10.3203	10.0377	10.6109

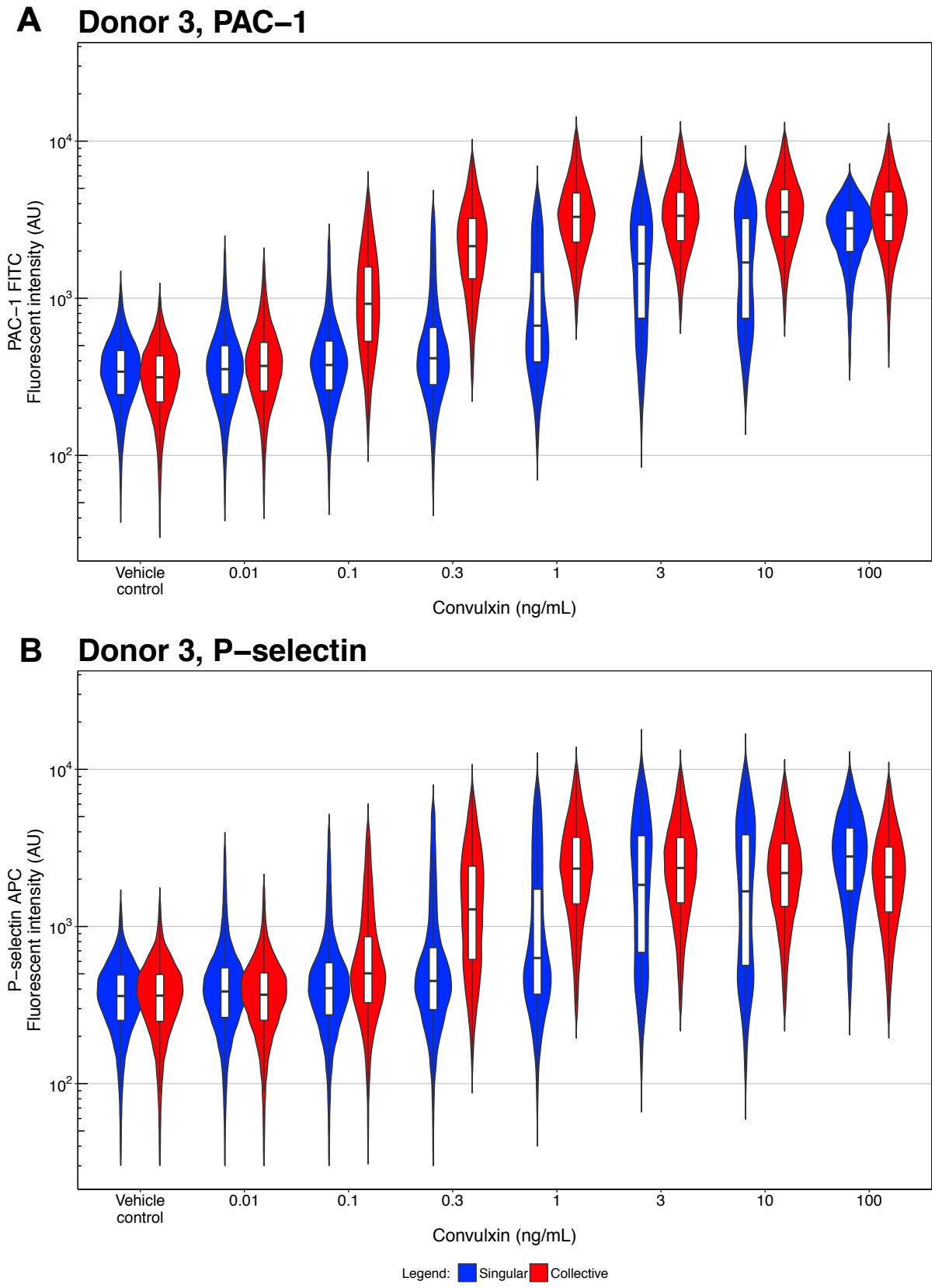


Figure B-3 Platelet responses of donor 3. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. This type of data is further discussed in section 3.3.2. N=8 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-4. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-4 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 3. This experiment was discussed in section 3.3.2. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9928	0.9908	0.9947	1.0022	0.9996	1.0048
0.01	0.9999	0.9968	1.0031	0.9616	0.9582	0.9650
0.1	1.7363	1.7151	1.7577	1.1424	1.1333	1.1516
0.3	5.4990	5.3343	5.6687	1.9877	1.9539	2.0221
1	22.3882	20.7723	24.1299	4.0836	3.9574	4.2137
3	13.2545	12.1638	14.4431	2.3804	2.2746	2.4913
10	14.0758	13.2570	14.9452	2.4598	2.3922	2.5293
100	1.5676	1.4500	1.6948	0.6123	0.5818	0.6445

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0289	1.0260	1.0318	1.0521	1.0484	1.0558
0.1	1.0484	1.0445	1.0523	1.0879	1.0828	1.0930
0.3	1.1471	1.1392	1.1551	1.1978	1.1882	1.2074
1	1.5275	1.5121	1.5431	1.5335	1.5178	1.5494
3	2.9193	2.8216	3.0203	2.8363	2.7431	2.9327
10	2.7956	2.7403	2.8519	2.5201	2.4740	2.5671
100	15.3546	14.3978	16.3751	8.7491	8.3438	9.1742

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0363	1.0338	1.0388	1.0095	1.0068	1.0122
0.1	1.8335	1.8119	1.8554	1.2401	1.2315	1.2487
0.3	6.3541	6.1683	6.5454	2.3757	2.3394	2.4125
1	34.4479	31.9828	37.1030	6.2485	6.0653	6.4372
3	38.9761	36.0200	42.1748	6.7370	6.5314	6.9491
10	39.6370	37.4578	41.9430	6.1855	6.0570	6.3168
100	24.2458	23.1997	25.3391	5.3457	5.2432	5.4501

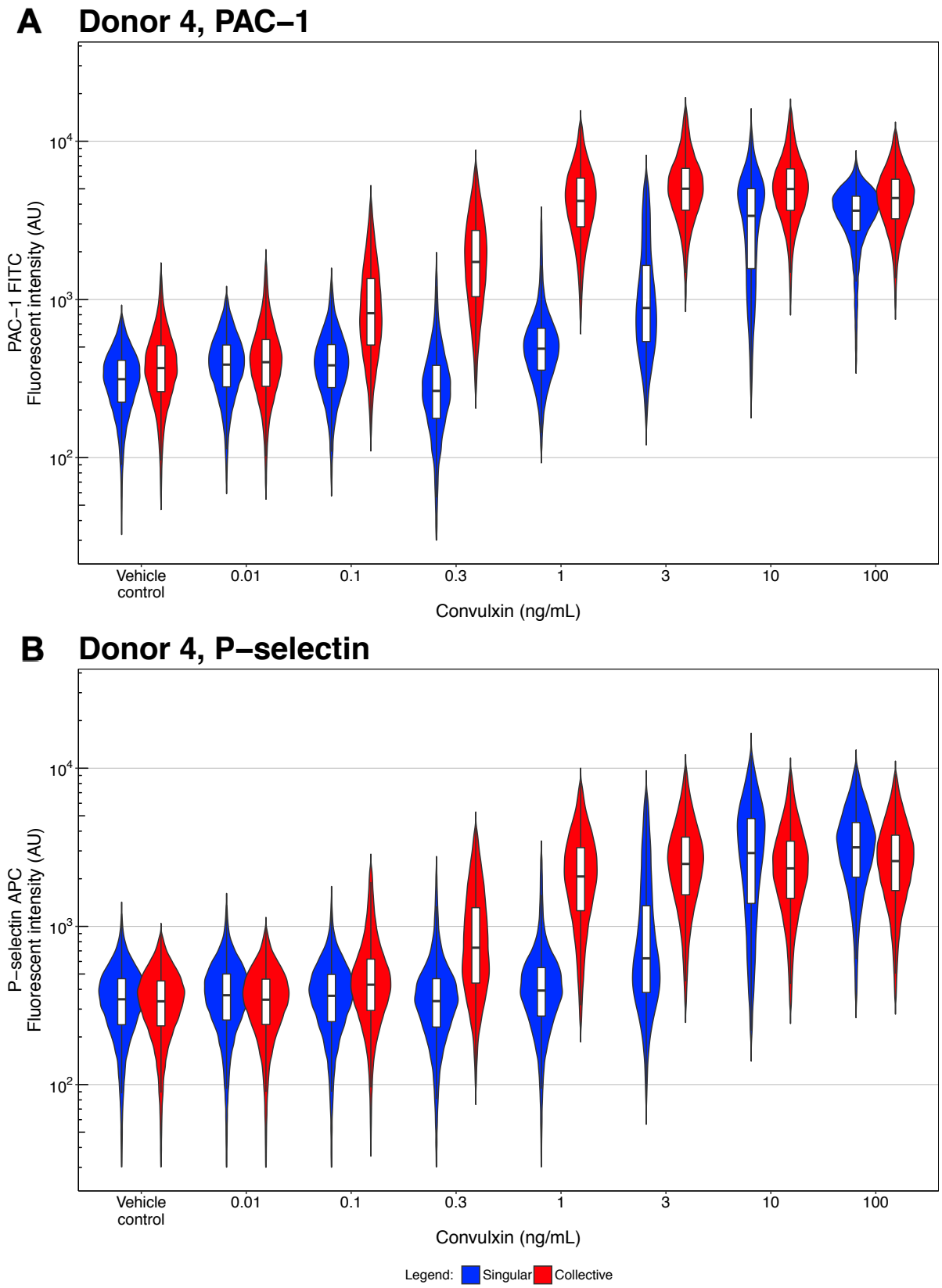


Figure B-4 Platelet responses of donor 4. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5.The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. This type of data is further discussed in section 3.3.2. N=8 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-5. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-5 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 4. This experiment was discussed in section 3.3.2. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	1.0298	1.0280	1.0316	0.9918	0.9904	0.9932
0.01	1.0403	1.0379	1.0428	0.9888	0.9871	0.9905
0.1	1.6136	1.6014	1.6258	1.0831	1.0793	1.0869
0.3	4.0644	3.9987	4.1312	1.5072	1.4953	1.5193
1	41.8842	39.4649	44.4519	5.2023	5.1030	5.3035
3	52.9249	48.4654	57.7946	5.9457	5.7894	6.1063
10	13.3207	12.1878	14.5590	1.5015	1.4482	1.5567
100	3.4703	3.1461	3.8281	0.7576	0.7153	0.8025

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0093	1.0079	1.0107	1.0064	1.0044	1.0084
0.1	1.0233	1.0210	1.0257	1.0073	1.0049	1.0097
0.3	1.0248	1.0213	1.0283	1.0293	1.0248	1.0338
1	1.0853	1.0802	1.0905	1.0457	1.0414	1.0499
3	1.7791	1.7614	1.7971	1.4761	1.4642	1.4880
10	6.5838	6.3899	6.7837	5.4268	5.2832	5.5744
100	21.0256	19.7404	22.3944	13.7568	13.0774	14.4715

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0196	1.0169	1.0223	1.0033	1.0022	1.0045
0.1	1.6033	1.5915	1.6152	1.1000	1.0968	1.1033
0.3	4.0446	3.9803	4.1100	1.5642	1.5536	1.5749
1	44.1414	41.5984	46.8398	5.4847	5.3820	5.5892
3	91.4323	83.7749	99.7896	8.8487	8.6264	9.0766
10	85.1604	78.3207	92.5974	8.2154	8.0190	8.4166
100	70.8519	65.7217	76.3826	10.5086	10.2249	10.8002

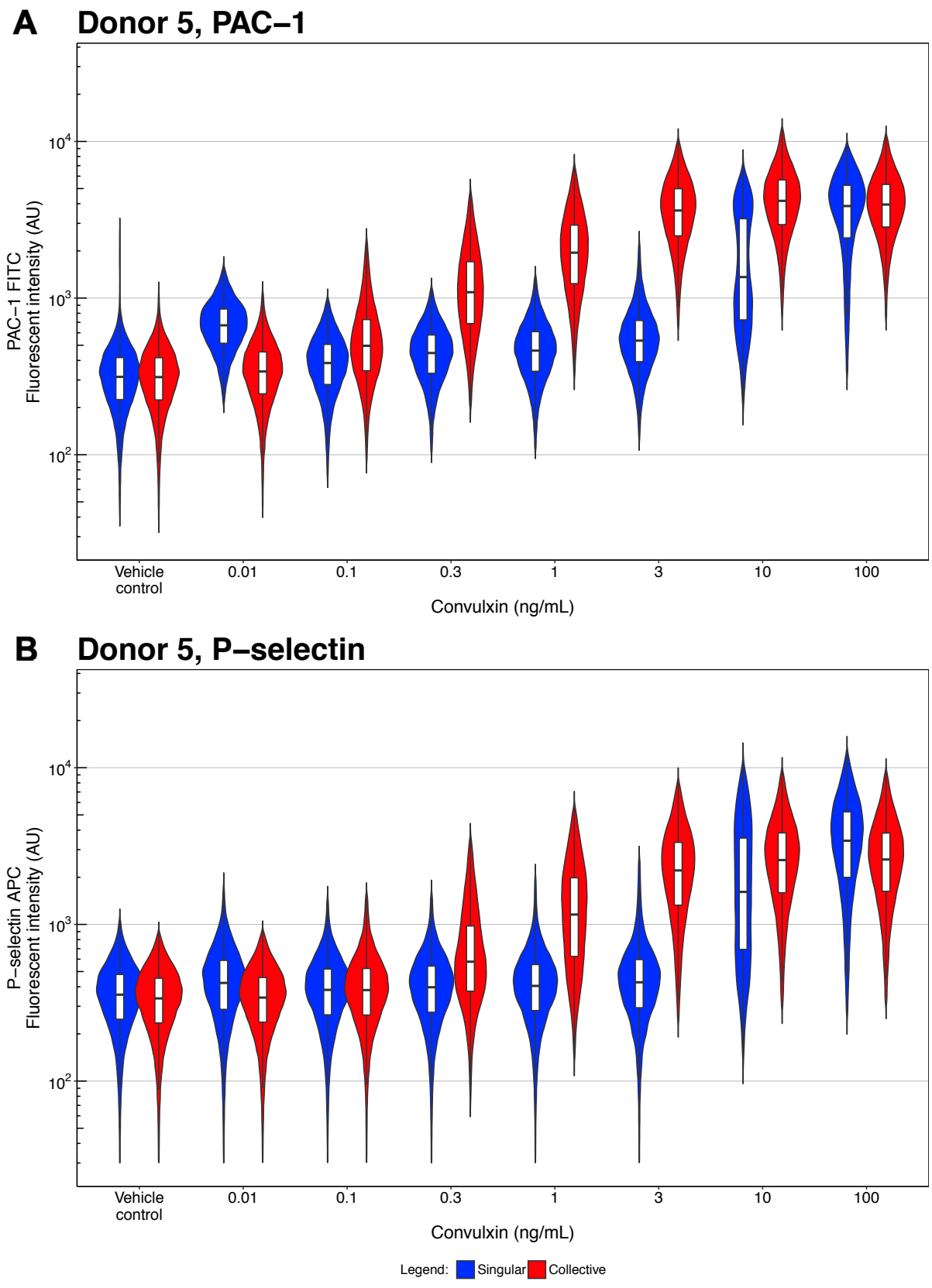


Figure B-5 Platelet responses of donor 5. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. This type of data is further discussed in section 3.3.2. N=8 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-6. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-6 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 5. This experiment was discussed in section 3.3.2. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9916	0.9899	0.9934	0.9927	0.9914	0.9941
0.01	0.8833	0.8775	0.8891	0.9620	0.9585	0.9656
0.1	1.1361	1.1320	1.1401	1.0112	1.0086	1.0139
0.3	2.1664	2.1450	2.1879	1.2831	1.2760	1.2901
1	5.7028	5.5902	5.8176	2.1937	2.1711	2.2165
3	29.6815	28.2224	31.2161	5.6037	5.4916	5.7181
10	19.6128	18.3943	20.9119	2.9932	2.9097	3.0790
100	4.6423	4.3159	4.9934	0.9250	0.8867	0.9651

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.1244	1.1169	1.1319	1.0327	1.0288	1.0366
0.1	0.9888	0.9870	0.9907	1.0141	1.0118	1.0164
0.3	1.0012	0.9990	1.0035	1.0216	1.0191	1.0241
1	1.0184	1.0160	1.0207	1.0310	1.0287	1.0332
3	1.0852	1.0808	1.0896	1.0614	1.0578	1.0649
10	2.5405	2.5044	2.5772	2.7387	2.6976	2.7803
100	10.7647	10.3735	11.1706	9.4573	9.1384	9.7874

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0016	1.0002	1.0030	1.0008	0.9998	1.0017
0.1	1.1328	1.1288	1.1368	1.0330	1.0311	1.0349
0.3	2.1873	2.1660	2.2089	1.3203	1.3136	1.3271
1	5.8565	5.7412	5.9741	2.2781	2.2551	2.3015
3	32.4828	30.8900	34.1577	5.9910	5.8725	6.1119
10	50.2469	47.2006	53.4898	8.2573	8.0619	8.4574
100	50.3945	47.3253	53.6628	8.8125	8.5955	9.0349

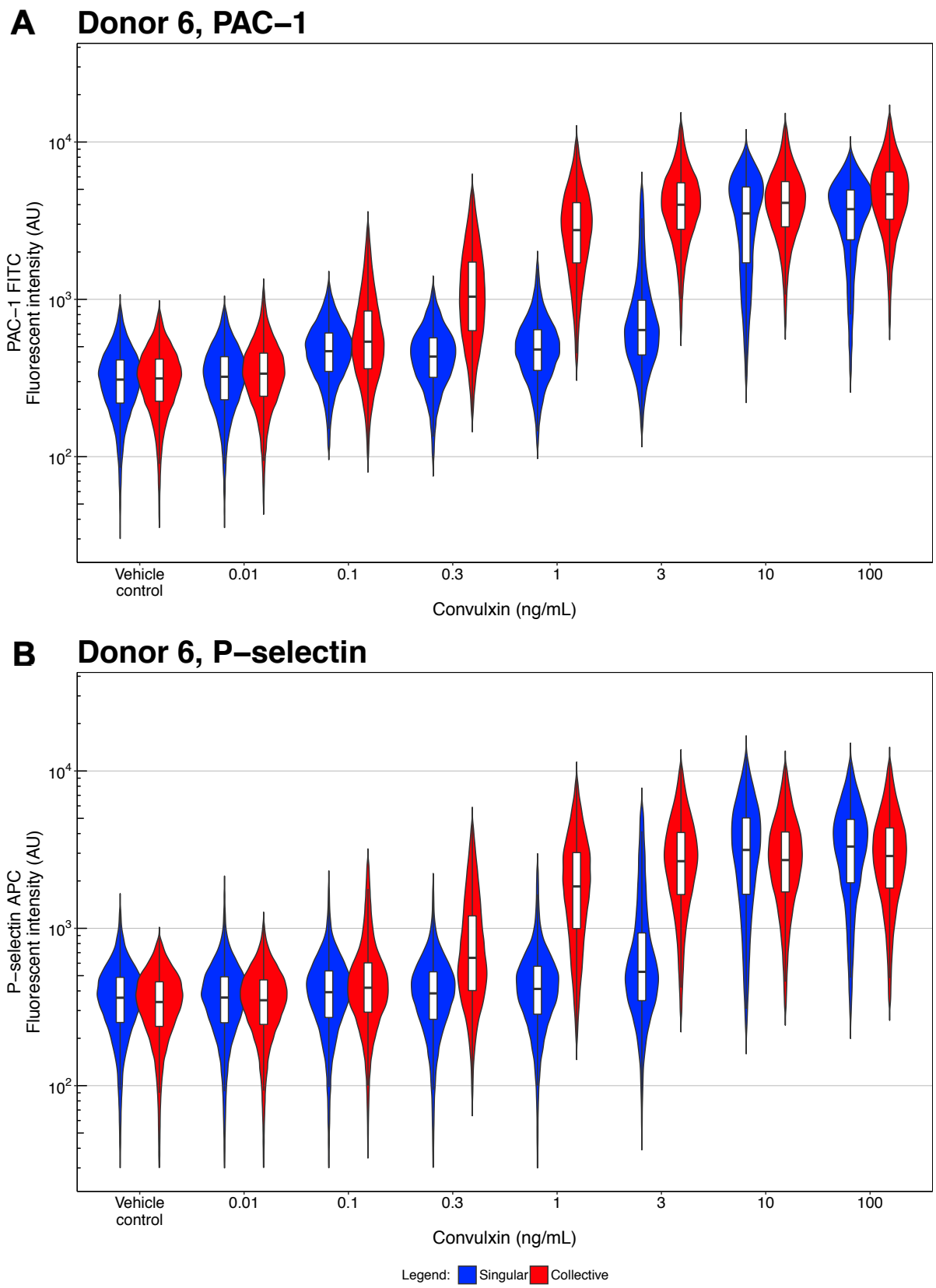


Figure B-6 Platelet responses of donor 6. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5.The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. This type of data is further discussed in section 3.3.2. N=8 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-7. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-7 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 6. This experiment was discussed in section 3.3.2. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9991	0.9979	1.0004	0.9851	0.9831	0.9872
0.01	1.0103	1.0088	1.0118	0.9894	0.9873	0.9916
0.1	1.1937	1.1878	1.1998	1.0698	1.0656	1.0740
0.3	2.0332	2.0139	2.0527	1.4221	1.4127	1.4316
1	9.5061	9.2503	9.7690	3.6964	3.6392	3.7545
3	27.6598	26.2053	29.1950	6.5047	6.3410	6.6727
10	5.2950	4.9584	5.6544	1.3488	1.2953	1.4045
100	3.8904	3.5940	4.2113	1.1215	1.0624	1.1838

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	0.9999	0.9985	1.0014	1.0031	1.0002	1.0059
0.1	1.0262	1.0234	1.0291	1.0130	1.0097	1.0163
0.3	1.0182	1.0155	1.0208	1.0108	1.0073	1.0142
1	1.0566	1.0532	1.0600	1.0361	1.0324	1.0398
3	1.3259	1.3165	1.3354	1.2878	1.2786	1.2970
10	7.7136	7.4604	7.9753	6.7681	6.5599	6.9830
100	11.5807	10.9863	12.2074	9.1505	8.7333	9.5876

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0111	1.0098	1.0125	1.0074	1.0062	1.0087
0.1	1.2261	1.2208	1.2315	1.1001	1.0968	1.1034
0.3	2.0719	2.0529	2.0912	1.4591	1.4502	1.4680
1	10.0528	9.7839	10.3291	3.8876	3.8284	3.9476
3	36.7071	34.7929	38.7268	8.5032	8.2968	8.7147
10	40.8789	38.6303	43.2584	9.2667	9.0309	9.5086
100	45.0932	42.5011	47.8434	10.4168	10.1355	10.7059

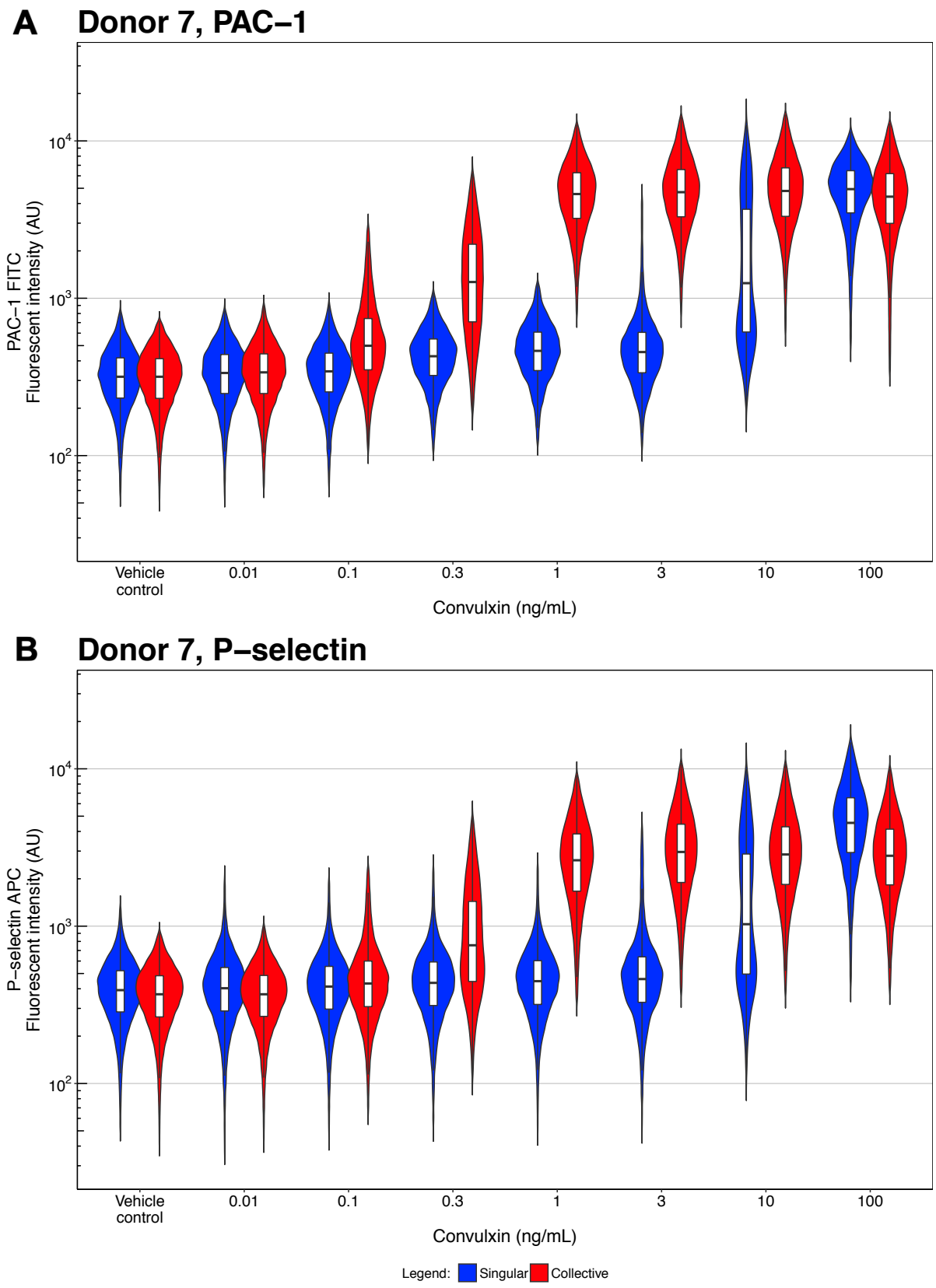


Figure B-7 Platelet responses of donor 7. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. This type of data is further discussed in section 3.3.2. N=8 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-8. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-8 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 7. This experiment was discussed in section 3.3.2. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9961	0.9951	0.9971	0.9850	0.9829	0.9871
0.01	1.0031	1.0019	1.0044	0.9733	0.9701	0.9766
0.1	1.1640	1.1594	1.1685	1.0486	1.0445	1.0527
0.3	2.4873	2.4595	2.5155	1.5529	1.5403	1.5655
1	56.8791	53.1322	60.8903	9.0659	8.8300	9.3080
3	57.9616	54.0365	62.1718	11.8475	11.4774	12.2296
10	19.2707	18.1501	20.4605	6.4027	6.1915	6.6212
100	0.6267	0.5569	0.7053	0.4730	0.4260	0.5252

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	0.9988	0.9974	1.0002	1.0160	1.0122	1.0199
0.1	1.0025	1.0009	1.0040	1.0184	1.0149	1.0219
0.3	1.0100	1.0077	1.0123	1.0307	1.0262	1.0352
1	1.0282	1.0257	1.0307	1.0316	1.0281	1.0352
3	1.0718	1.0656	1.0781	1.0823	1.0747	1.0899
10	2.2478	2.2147	2.2815	1.9802	1.9536	2.0072
100	33.0097	29.5394	36.8876	26.5037	23.9812	29.2916

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0058	1.0050	1.0067	1.0040	1.0028	1.0051
0.1	1.1714	1.1670	1.1758	1.0841	1.0811	1.0872
0.3	2.5221	2.4943	2.5501	1.6249	1.6133	1.6366
1	58.7102	54.8445	62.8483	9.4952	9.2495	9.7474
3	62.3676	58.1579	66.8820	13.0177	12.6201	13.4279
10	43.4859	41.0337	46.0848	12.8719	12.4817	13.2742
100	20.7685	19.9486	21.6221	12.7274	12.3374	13.1297

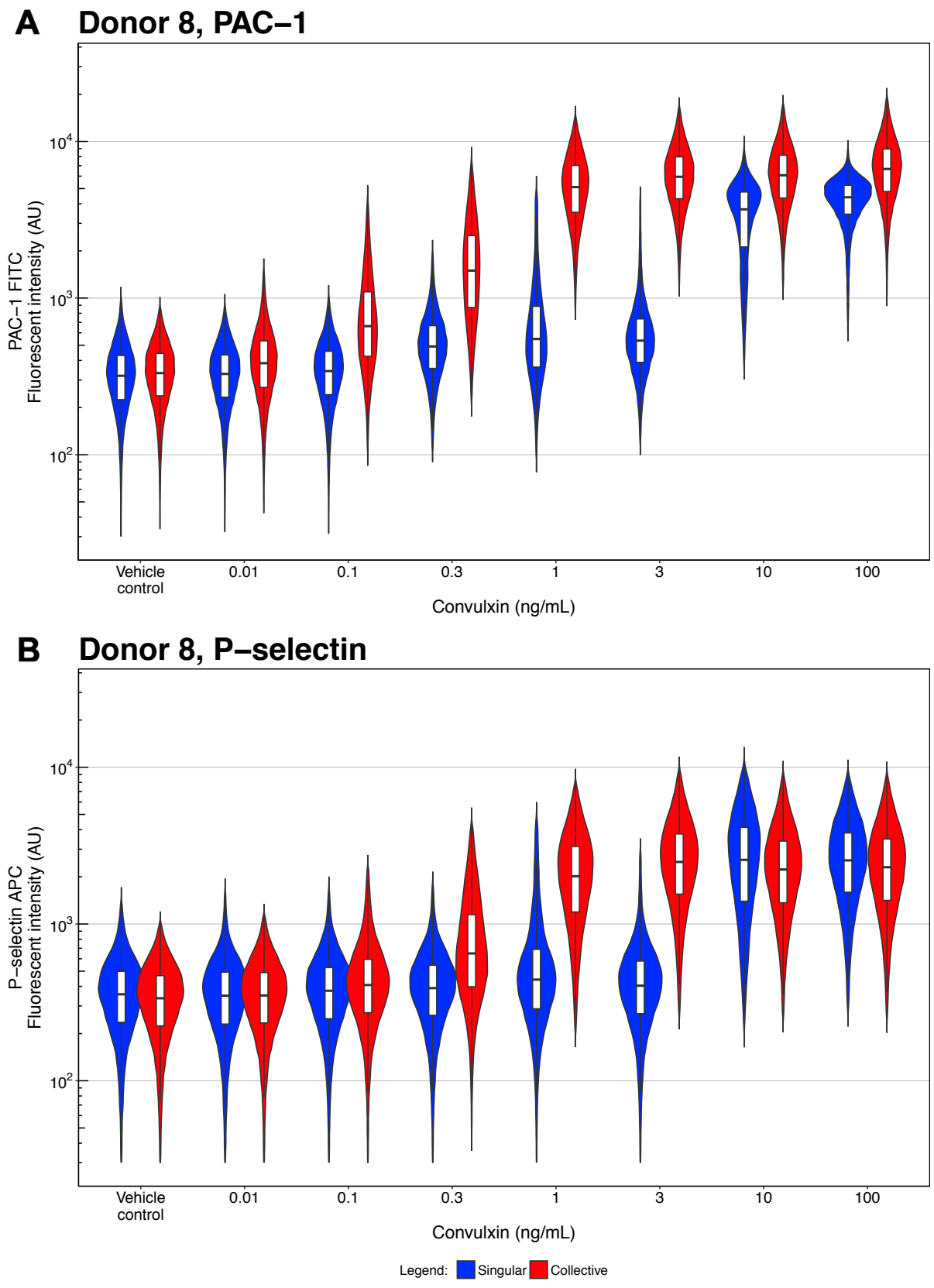


Figure B-8 Platelet responses of donor 8. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. This type of data is further discussed in section 3.3.2. N=8 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-8. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-9 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 8. This experiment was discussed in section 3.3.2. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9959	0.9945	0.9973	0.9844	0.9822	0.9866
0.01	1.0300	1.0279	1.0320	0.9890	0.9867	0.9913
0.1	1.3948	1.3868	1.4029	1.0451	1.0417	1.0485
0.3	2.9667	2.9271	3.0068	1.3812	1.3727	1.3898
1	57.8229	53.6290	62.3447	4.3430	4.2637	4.4238
3	121.4011	109.4546	134.6514	7.5166	7.3413	7.6960
10	11.5982	10.4266	12.9013	1.0864	1.0473	1.1270
100	2.5141	2.2319	2.8320	0.8156	0.7835	0.8489

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	0.9978	0.9962	0.9994	1.0018	0.9989	1.0047
0.1	1.0018	1.0001	1.0034	1.0086	1.0057	1.0115
0.3	1.0688	1.0655	1.0721	1.0148	1.0119	1.0177
1	1.2561	1.2490	1.2633	1.1440	1.1384	1.1496
3	1.1276	1.1226	1.1326	1.0387	1.0350	1.0425
10	10.5518	10.1203	11.0016	5.8812	5.7061	6.0616
100	41.3602	38.2605	44.7111	8.4068	8.1277	8.6955

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0319	1.0300	1.0338	1.0065	1.0050	1.0080
0.1	1.4030	1.3950	1.4110	1.0708	1.0680	1.0737
0.3	3.1839	3.1424	3.2260	1.4239	1.4155	1.4324
1	72.9334	67.6572	78.6211	5.0473	4.9577	5.1384
3	137.4542	123.9387	152.4435	7.9316	7.7480	8.1196
10	122.8853	111.4171	135.5340	6.4906	6.3565	6.6276
100	104.4126	95.4189	114.2539	6.9650	6.8153	7.1180

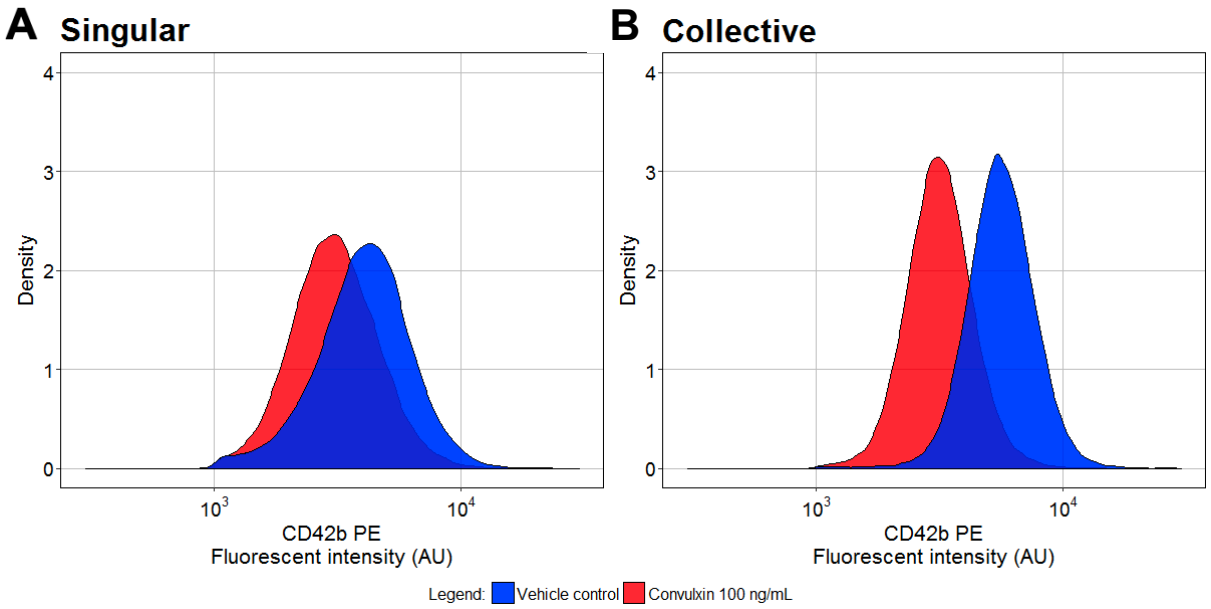


Figure B-9 Density plots of the CD42b (GPIb) expression. Comparing CD42b expression for active (convulxin 100 ng/mL, red) and non-active (vehicle control, blue) platelets **A)** in droplets and **B)** in suspension. This type of data is further discussed in section 3.3.4.

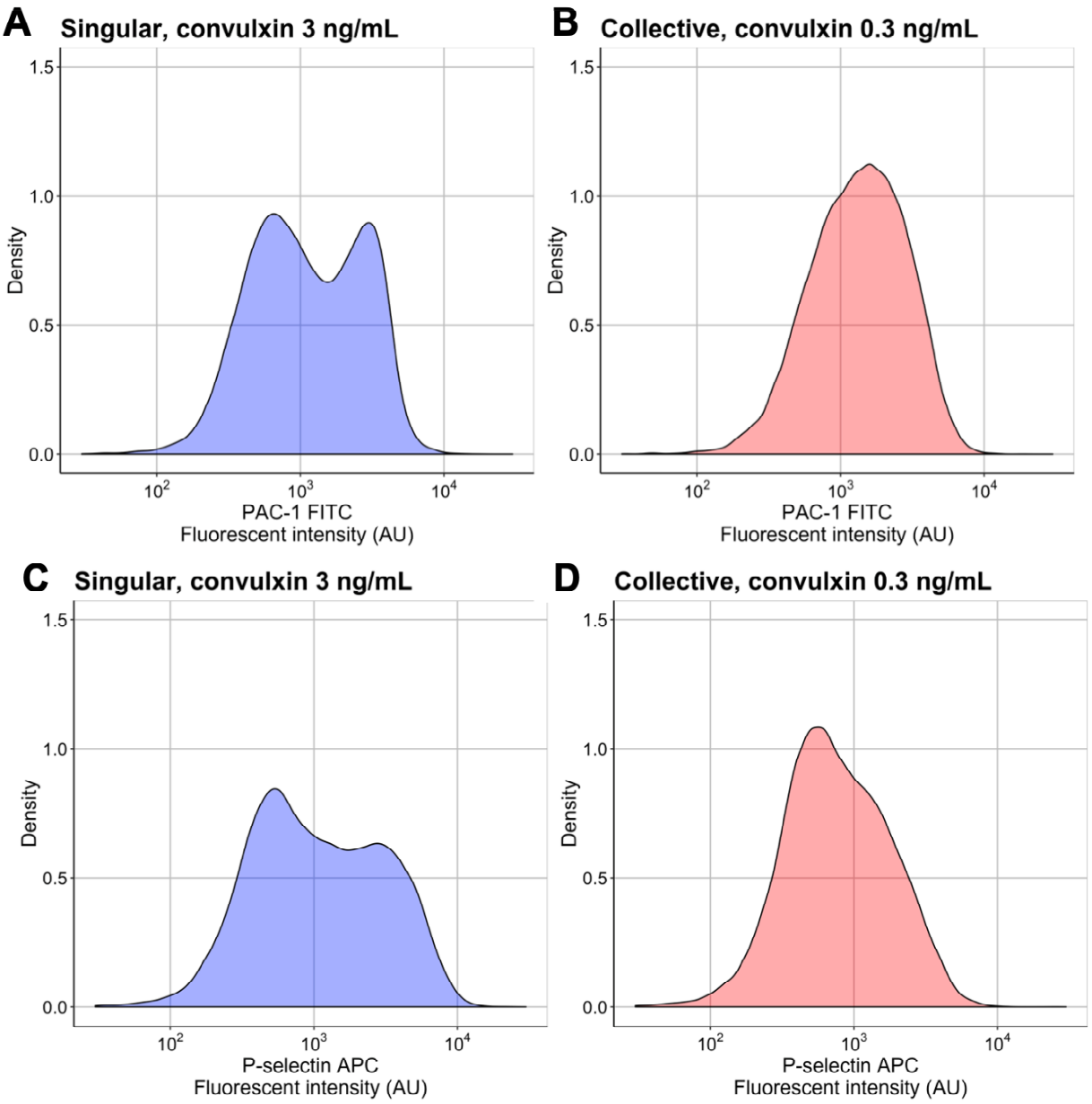


Figure B-10 Density plot of the intermediate platelet response to convulxin. Intermediate response shown for **(A and C)** single platelets to 3 ng/mL convulxin and **(B and D)** collective platelets to 0.3 ng/mL convulxin. Samples from donor 1 and same day. This type of data is further discussed in section 3.3.5.

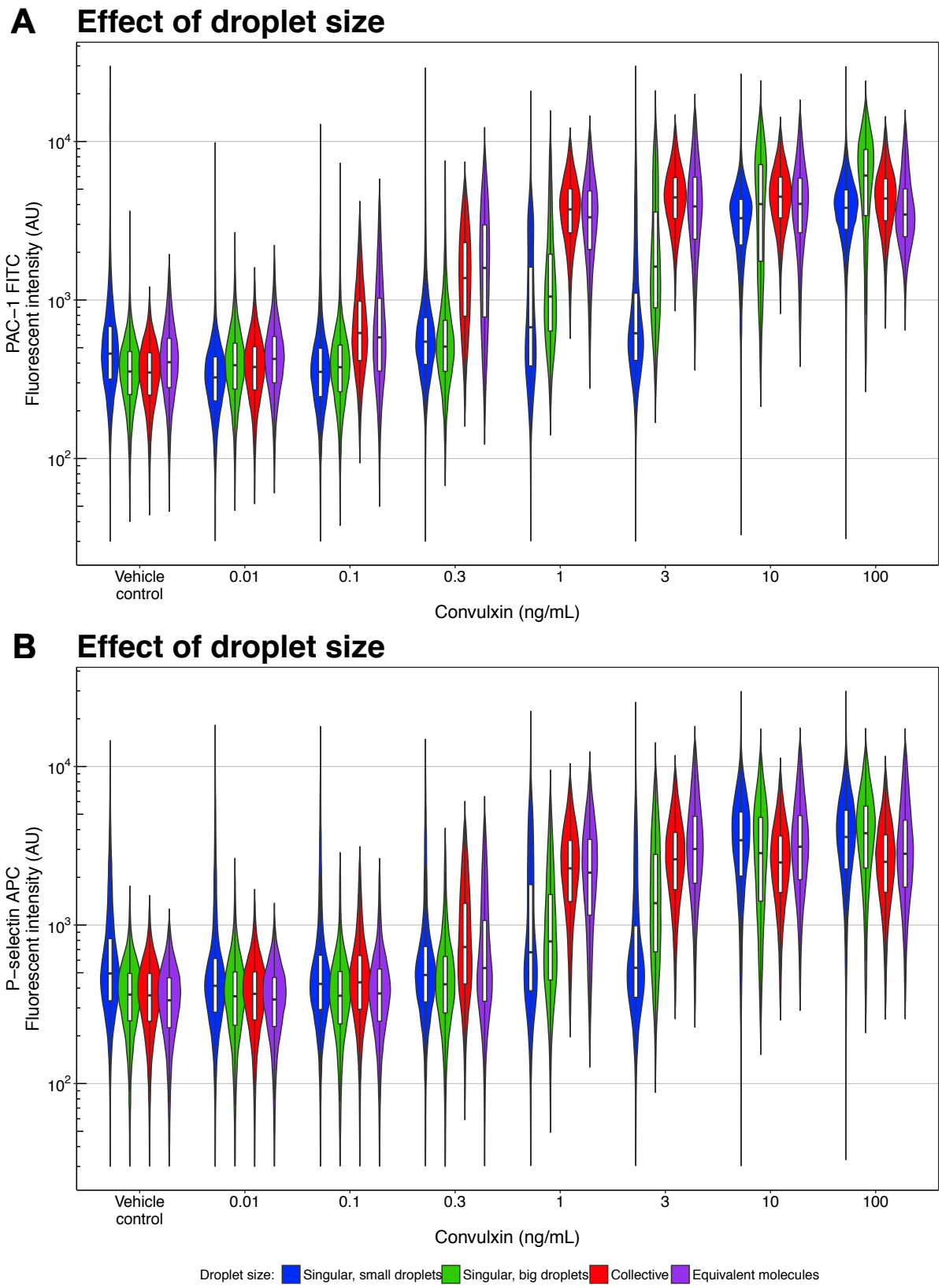


Figure B-11 Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to convulxin stimulation in standard droplets of ~25 μm diameter (singular, small droplets, blue), in bigger droplets of ~55 μm diameter (singular, big droplets, green), in suspension (collective, red) or in suspension with a 25 times higher concentration of platelets (equivalent molecules, purple), to correct for convulxin molecules found in empty droplets. These were not measured on the same day but it is the same donor (donor 1). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. This type of data is further discussed in section 3.3.5. N=8 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-10. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-10 Relative activation and confidence intervals of dose response relationship of platelet activity in response to convulxin. Platelets were stimulated in standard droplets of ~25 μm diameter (singular, small droplets), in bigger droplets of ~55 μm diameter (singular, big droplets), in suspension (collective) or in suspension with a 25 times higher concentration of platelets (equivalent molecules), to correct for convulxin molecules found in empty droplets. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 1, measurements on different days. This experiment was discussed in section 3.3.5. Top table shows the relative activation (RA) of singular, small droplets compared to singular, big droplets response, second table shows the RA of vehicle control compared to convulxin stimulation for singular platelets in big droplets, third table the RA of collective compared to equivalent molecules response and the bottom table shows the RA of vehicle control compared to convulxin stimulation for equivalent molecules. Also see Table B-2.

Singular small droplets vs singular big droplets response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	1.0028	1.0000	1.0056	0.9574	0.9540	0.9609
0.01	0.9838	0.9798	0.9879	0.9127	0.9081	0.9172
0.1	1.0027	0.9987	1.0068	0.9128	0.9075	0.9182
0.3	1.0922	1.0844	1.1001	0.9633	0.9562	0.9704
1	1.7688	1.7401	1.7979	1.3804	1.3618	1.3992
3	1.5812	1.5399	1.6236	1.2877	1.2585	1.3175
10	6.7518	6.4567	7.0604	5.1096	4.9252	5.3008
100	0.5184	0.4759	0.5647	0.9664	0.9042	1.0327

Singular big droplets vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0189	1.0150	1.0229	1.0173	1.0136	1.0210
0.1	1.0192	1.0154	1.0230	1.0216	1.0179	1.0254
0.3	1.1343	1.1267	1.1420	1.0878	1.0816	1.0940
1	2.0535	2.0219	2.0856	1.6520	1.6318	1.6724
3	3.2374	3.1668	3.3096	2.5145	2.4692	2.5607
10	8.1514	7.7977	8.5211	5.8360	5.6270	6.0528
100	13.9727	13.2340	14.7527	11.9965	11.4119	12.6111

Collective vs equivalent molecules response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	1.0481	1.0456	1.0506	0.9918	0.9902	0.9934
0.01	1.0355	1.0329	1.0382	0.9882	0.9864	0.9900
0.1	1.0220	1.0145	1.0296	0.9443	0.9408	0.9479
0.3	1.0583	1.0400	1.0770	0.8662	0.8587	0.8738
1	0.3152	0.2955	0.3362	0.6938	0.6753	0.7130
3	0.1945	0.1769	0.2140	1.0823	1.0415	1.1248
10	0.2306	0.2100	0.2533	1.4221	1.3657	1.4808
100	1.0060	0.9094	1.1127	1.0989	1.0578	1.1416

Equivalent molecules vehicle control vs convulxin response						
	PAC-1			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0001	0.9970	1.0032	1.0020	1.0006	1.0035
0.1	1.2818	1.2745	1.2892	1.0446	1.0422	1.0471
0.3	2.8324	2.7965	2.8688	1.3590	1.3514	1.3665
1	12.2047	11.8337	12.5874	4.5497	4.4733	4.6275
3	17.7957	17.1351	18.4817	10.5568	10.2671	10.8546
10	19.8788	19.1004	20.6890	13.1170	12.7139	13.5328
100	61.1766	56.9592	65.7062	10.4308	10.1464	10.7232

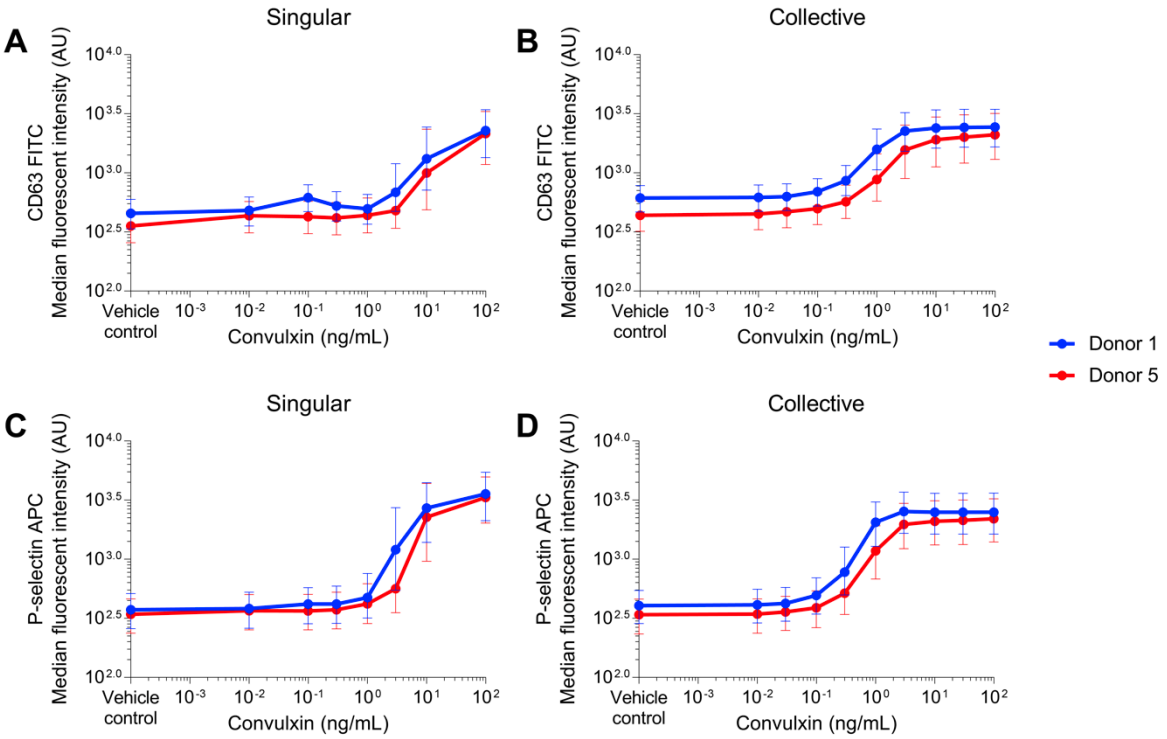


Figure B-12 A dose-response curve of convulxin and median fluorescent intensity of the **(A-B)** CD63 and **(C-D)** P-selectin antibody binding. Platelets were activated **(A and C)** singular, within droplets and (**B and D)** collective, in suspension. The point on the Y-axis is the vehicle control. Convulxin is used at concentrations of 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 and 100 ng/mL (singular response was not measured with 0.03 and 30 ng/mL). Results are shown as median with 25 and 75 percentiles. This type of data is further discussed in section 3.3.6.

Table B-11 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with CD63 antibody and P-selectin antibody, blood from donor 1. This experiment was discussed in section 3.3.6. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	CD63			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	1.0630	1.0596	1.0664	0.9951	0.9932	0.9971
0.01	1.0627	1.0592	1.0661	0.9907	0.9887	0.9927
0.1	1.0892	1.0836	1.0948	1.0681	1.0643	1.0719
0.3	1.4747	1.4639	1.4856	1.4654	1.4544	1.4764
1	4.2857	4.2118	4.3608	5.1177	5.0107	5.2271
3	7.2495	7.0428	7.4622	4.7032	4.5577	4.8534
10	4.9908	4.8222	5.1654	1.7515	1.6833	1.8225
100	2.1376	2.0460	2.2333	1.0267	0.9791	1.0766

Singular vehicle control vs convulxin response						
	CD63			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0073	1.0049	1.0098	1.0096	1.0073	1.0119
0.1	1.0775	1.0737	1.0813	1.0195	1.0170	1.0220
0.3	1.0396	1.0362	1.0430	1.0565	1.0529	1.0601
1	1.0501	1.0462	1.0540	1.1841	1.1774	1.1908
3	1.4093	1.3968	1.4220	2.2102	2.1777	2.2433
10	2.4585	2.4193	2.4984	5.8087	5.6437	5.9786
100	6.3924	6.2072	6.5831	10.2143	9.8309	10.6126

Collective vehicle control vs convulxin response						
	CD63			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0070	1.0031	1.0110	1.0051	1.0034	1.0067
0.1	1.1041	1.0987	1.1095	1.0943	1.0908	1.0978
0.3	1.4422	1.4317	1.4528	1.5558	1.5450	1.5666
1	4.2336	4.1611	4.3074	6.0895	5.9661	6.2154
3	9.6117	9.3491	9.8815	10.4461	10.1599	10.7403
10	11.5430	11.1956	11.9012	10.2239	9.9474	10.5082
100	12.8550	12.4423	13.2814	10.5383	10.2463	10.8387

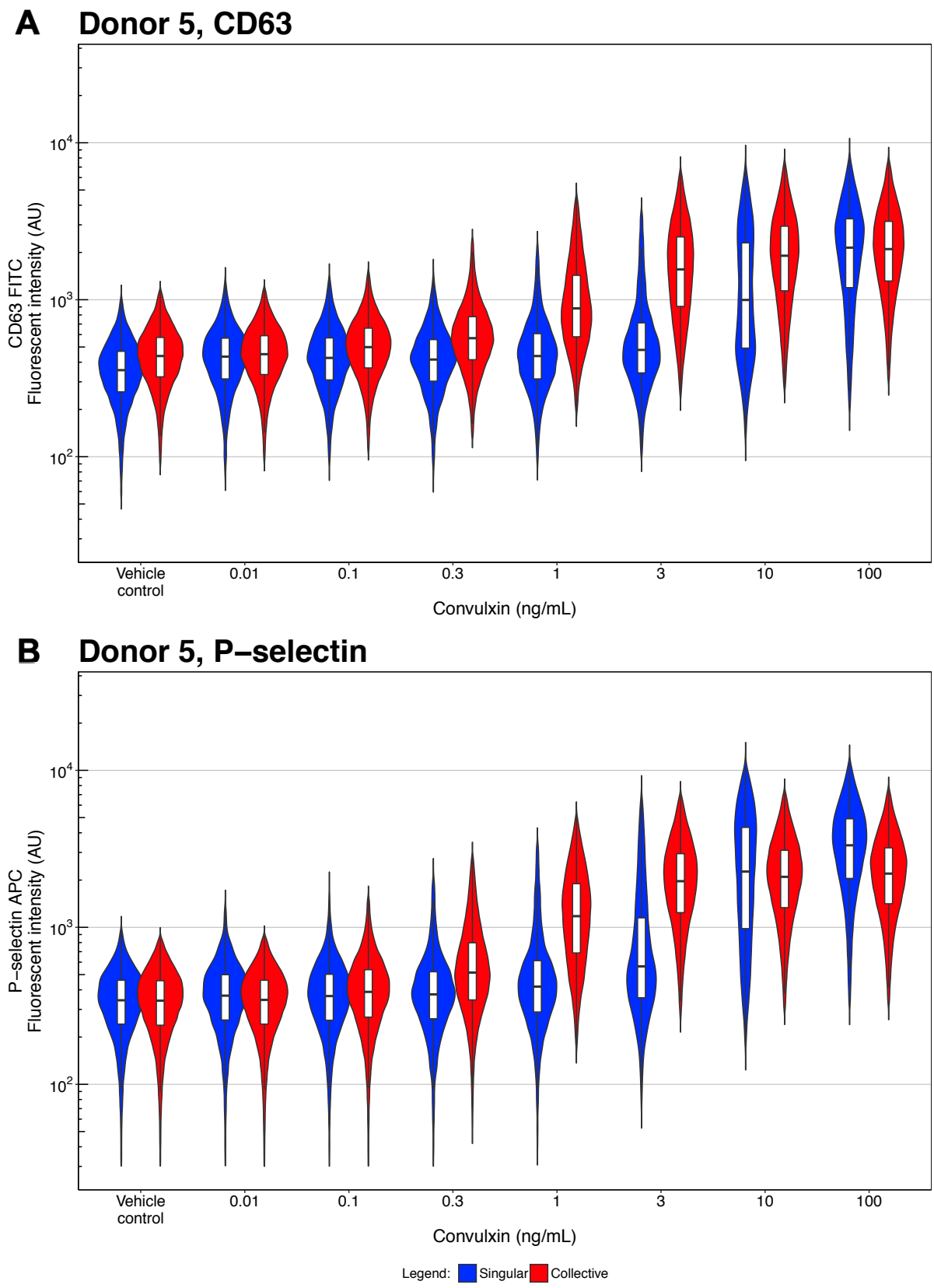


Figure B-13 Platelet responses of donor 5. Violin and box plots of the fluorescent intensity observed with **A)** CD63 or **B)** P-selectin antibody in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD61 and following the gating procedure described in section 2.3.5. The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. This type of data is further discussed in section 3.3.6. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-12. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-12 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with CD63 antibody and P-selectin antibody, blood from donor 5. This experiment was discussed in section 3.3.6. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	CD63			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	1.0110	1.0087	1.0133	0.9949	0.9931	0.9966
0.01	0.9962	0.9914	1.0010	0.9829	0.9788	0.9870
0.1	1.0197	1.0136	1.0258	1.0100	1.0046	1.0155
0.3	1.1070	1.0996	1.1145	1.1367	1.1272	1.1464
1	1.6113	1.5973	1.6254	2.1157	2.0910	2.1407
3	2.9043	2.8622	2.9471	4.0075	3.9257	4.0910
10	2.3912	2.3363	2.4474	1.6774	1.6222	1.7346
100	1.3142	1.2647	1.3657	0.6461	0.6092	0.6852

Singular vehicle control vs convulxin response						
	CD63			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0168	1.0117	1.0219	1.0130	1.0086	1.0175
0.1	1.0250	1.0190	1.0312	1.0201	1.0147	1.0255
0.3	1.0248	1.0186	1.0311	1.0500	1.0420	1.0580
1	1.0700	1.0651	1.0749	1.1217	1.1155	1.1279
3	1.1576	1.1514	1.1639	1.3978	1.3867	1.4090
10	1.9753	1.9447	2.0065	3.8211	3.7217	3.9231
100	4.7565	4.6039	4.9142	11.4754	10.8690	12.1156

Collective vehicle control vs convulxin response						
	CD63			P-selectin		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0019	1.0000	1.0038	1.0008	1.0000	1.0016
0.1	1.0338	1.0312	1.0364	1.0356	1.0337	1.0375
0.3	1.1221	1.1180	1.1262	1.1996	1.1948	1.2045
1	1.7053	1.6921	1.7186	2.3852	2.3603	2.4104
3	3.3255	3.2800	3.3716	5.6303	5.5238	5.7388
10	4.6718	4.5917	4.7534	6.4425	6.3101	6.5776
100	6.1828	6.0575	6.3108	7.4518	7.2855	7.6218

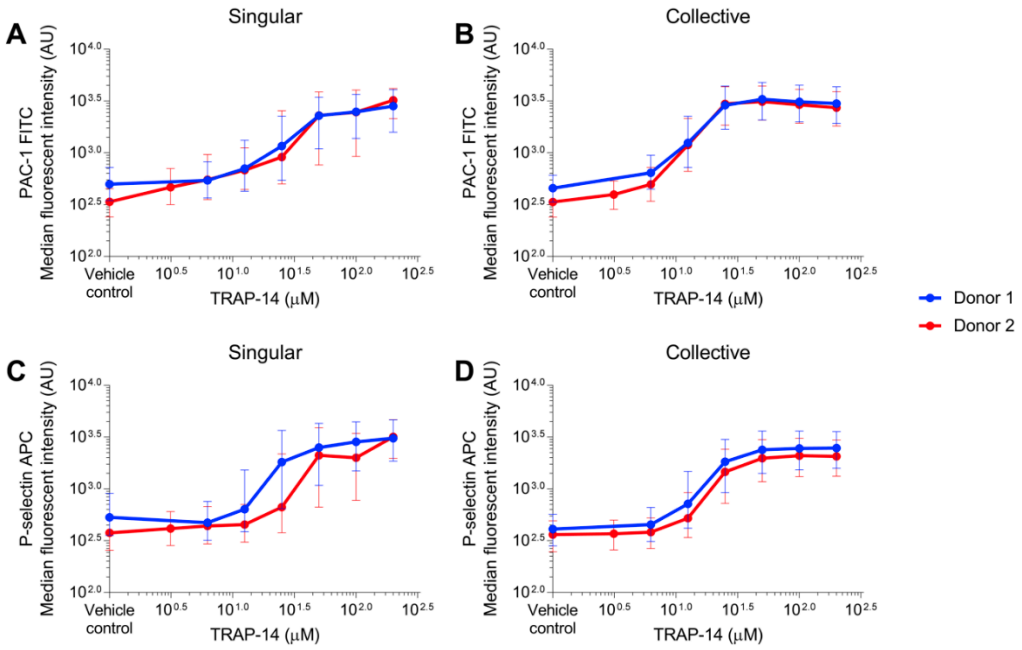


Figure B-14 A dose-response curve of TRAP-14 and median fluorescent intensity of the **(A-B)** PAC-1 and **(C-D)** P-selectin antibody binding. Platelets were activated **(A and C)** singular, within droplets and (**B and D)** collective, in suspension. The point on the Y-axis is the vehicle control. TRAP-14 is used at concentrations of 3.125 (only donor 2), 6.25, 12.5, 25, 50, 100 and 200 μM. Results are shown as median with 25 and 75 percentiles. This type of data is further discussed in section 3.3.7.

Table B-13 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to TRAP-14. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 1. This experiment was discussed in section 3.3.7. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to TRAP-14 stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to TRAP-14 stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
TRAP-14 (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9104	0.9050	0.9158	0.8225	0.8158	0.8293
6.25	1.0673	1.0591	1.0755	0.9266	0.9202	0.9330
12.5	1.6542	1.6291	1.6796	1.0473	1.0341	1.0607
25	4.2423	4.1155	4.3730	1.2793	1.2487	1.3107
50	3.4479	3.2970	3.6057	1.5296	1.4763	1.5848
100	2.4146	2.2644	2.5749	1.2554	1.1818	1.3336
200	2.0947	1.9894	2.2054	1.0004	0.9490	1.0547

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
TRAP-14 (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
6.25	1.0570	1.0484	1.0657	0.9504	0.9409	0.9600
12.5	1.3382	1.3223	1.3542	1.1953	1.1796	1.2112
25	1.9367	1.9049	1.9691	2.2268	2.1807	2.2738
50	3.8764	3.7620	3.9942	3.3103	3.2134	3.4102
100	5.3873	5.0975	5.6936	4.8932	4.6255	5.1765
200	6.0026	5.7654	6.2496	6.9590	6.6384	7.2950

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
TRAP-14 (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
6.25	1.2392	1.2328	1.2455	1.0706	1.0665	1.0748
12.5	2.4314	2.4043	2.4589	1.5219	1.5109	1.5330
25	9.0247	8.7923	9.2632	3.4633	3.4124	3.5150
50	14.6808	14.1933	15.1850	6.1559	6.0277	6.2868
100	14.2886	13.8211	14.7719	7.4684	7.2948	7.6461
200	13.8108	13.3673	14.2691	8.4641	8.2534	8.6802

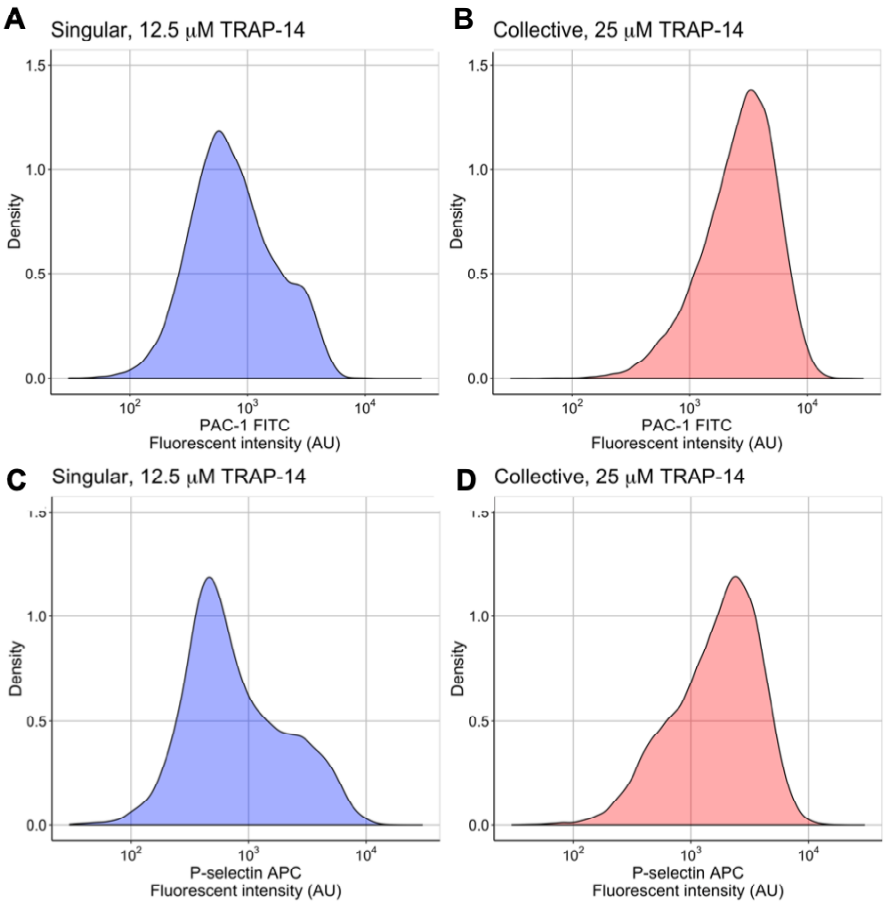


Figure B-15 Density plot of the intermediate platelet response to TRAP-14. Intermediate response shown for **(A and C)** single platelets to 12.5 μM TRAP-14 and **(B and D)** collective platelets to 25 μM TRAP-14. Samples from donor 1 and same day. This type of data is further discussed in section 3.3.7.

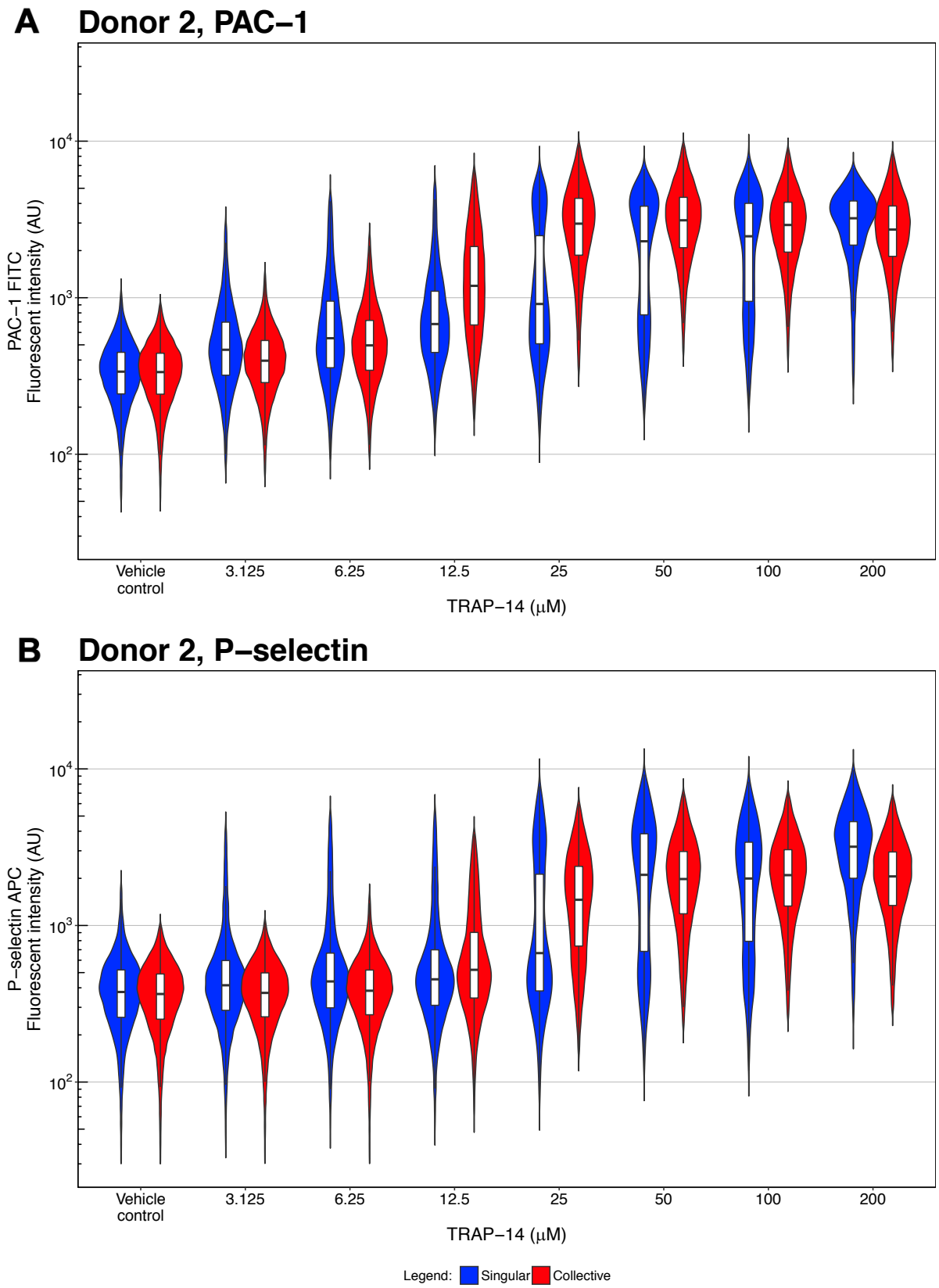


Figure B-16 Platelet responses of donor 2. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to TRAP-14 stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. This type of data is further discussed in section 3.3.7. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-14. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-14 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to TRAP-14. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 2. This experiment was discussed in section 3.3.7. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to TRAP-14 stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to TRAP-14 stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
TRAP-14 (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9946	0.9928	0.9965	0.9742	0.9712	0.9771
6.25	0.8624	0.8541	0.8707	0.8737	0.8676	0.8799
12.5	1.6715	1.6475	1.6959	1.0835	1.0746	1.0926
25	5.8258	5.6441	6.0133	1.7315	1.7021	1.7614
50	5.4746	5.2543	5.7041	1.6402	1.5979	1.6835
100	4.0582	3.8877	4.2362	1.9220	1.8620	1.9838
200	1.0893	0.9924	1.1957	0.5987	0.5499	0.6517

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
TRAP-14 (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
6.25	1.2942	1.2823	1.3062	1.1366	1.1282	1.1451
12.5	1.4001	1.3862	1.4142	1.1510	1.1427	1.1595
25	1.8536	1.8278	1.8797	1.5887	1.5694	1.6083
50	3.0894	3.0310	3.1490	2.9494	2.8941	3.0058
100	3.6992	3.6033	3.7977	3.1689	3.0935	3.2462
200	12.1330	11.1143	13.2451	10.5419	9.7091	11.4461

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
TRAP-14 (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
6.25	1.1221	1.1183	1.1259	1.0194	1.0176	1.0213
12.5	2.3530	2.3281	2.3781	1.2803	1.2739	1.2867
25	10.8568	10.5519	11.1704	2.8238	2.7892	2.8589
50	17.0047	16.3967	17.6352	4.9658	4.8763	5.0569
100	15.0933	14.5889	15.6150	6.2521	6.1234	6.3835
200	13.2880	12.8745	13.7148	6.4784	6.3428	6.6170

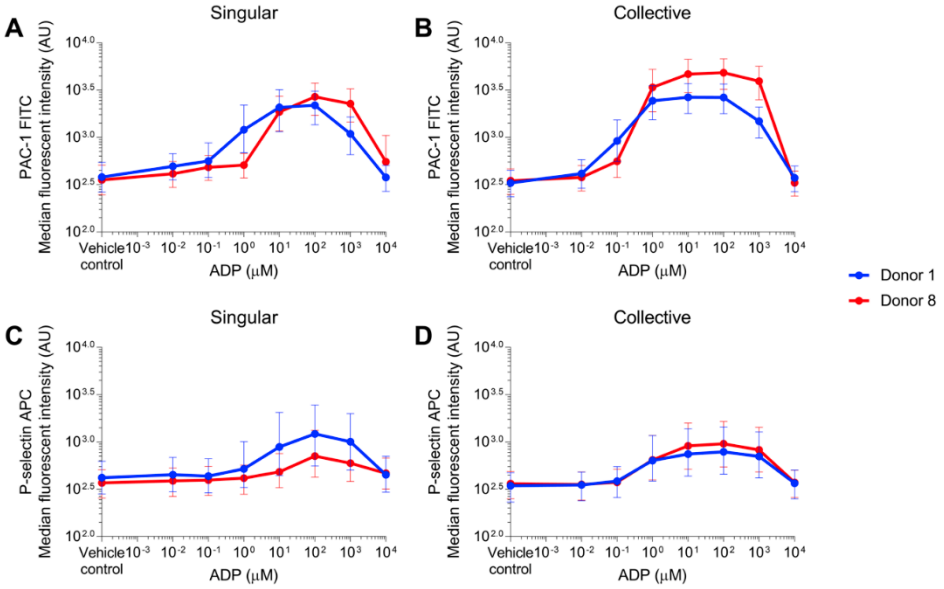


Figure B-17 Dose-response curves of ADP and median fluorescent intensity of the **(A-B)** PAC-1 and **(C-D)** P-selectin antibody binding. Platelets were activated **(A and C)** singular, within droplets and (**B and D)** collective, in suspension. The point on the Y-axis is the vehicle control. ADP is used at concentrations of 0.01, 0.1, 1, 10, 100, 1000 and 10000 μM. Results are shown as median with 25 and 75 percentiles. This type of data is further discussed in section 3.3.8.

Table B-15 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to ADP. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 1. This experiment was discussed in section 3.3.8. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to ADP stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to ADP stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
ADP (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9466	0.9440	0.9492	0.8955	0.8924	0.8987
0.01	0.9732	0.9697	0.9767	0.8629	0.8591	0.8667
0.1	1.4745	1.4607	1.4883	0.9161	0.9120	0.9202
1	3.4784	3.3840	3.5755	1.0778	1.0683	1.0874
10	2.5166	2.4271	2.6093	0.8581	0.8479	0.8684
100	1.9326	1.8573	2.0110	0.7202	0.7099	0.7306
1000	1.7571	1.7175	1.7976	0.7602	0.7476	0.7731
10000	1.0025	1.0010	1.0040	0.8476	0.8429	0.8523

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
ADP (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0115	1.0076	1.0154	1.0424	1.0367	1.0481
0.1	1.1633	1.1571	1.1695	1.0350	1.0298	1.0403
1	2.2407	2.2102	2.2716	1.1867	1.1779	1.1955
10	4.5366	4.4433	4.6318	1.6544	1.6374	1.6716
100	6.1232	5.9668	6.2836	2.0386	2.0125	2.0650
1000	2.0711	2.0357	2.1072	1.7815	1.7534	1.8100
10000	0.9412	0.9386	0.9439	1.0586	1.0519	1.0654

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
ADP (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0399	1.0375	1.0423	1.0044	1.0031	1.0058
0.1	1.8119	1.7972	1.8268	1.0588	1.0561	1.0614
1	8.2337	8.0382	8.4339	1.4282	1.4198	1.4368
10	12.0605	11.7070	12.4246	1.5852	1.5743	1.5963
100	12.5014	12.1280	12.8861	1.6393	1.6275	1.6512
1000	3.8445	3.7868	3.9031	1.5123	1.5025	1.5221
10000	0.9968	0.9954	0.9983	1.0019	1.0006	1.0032

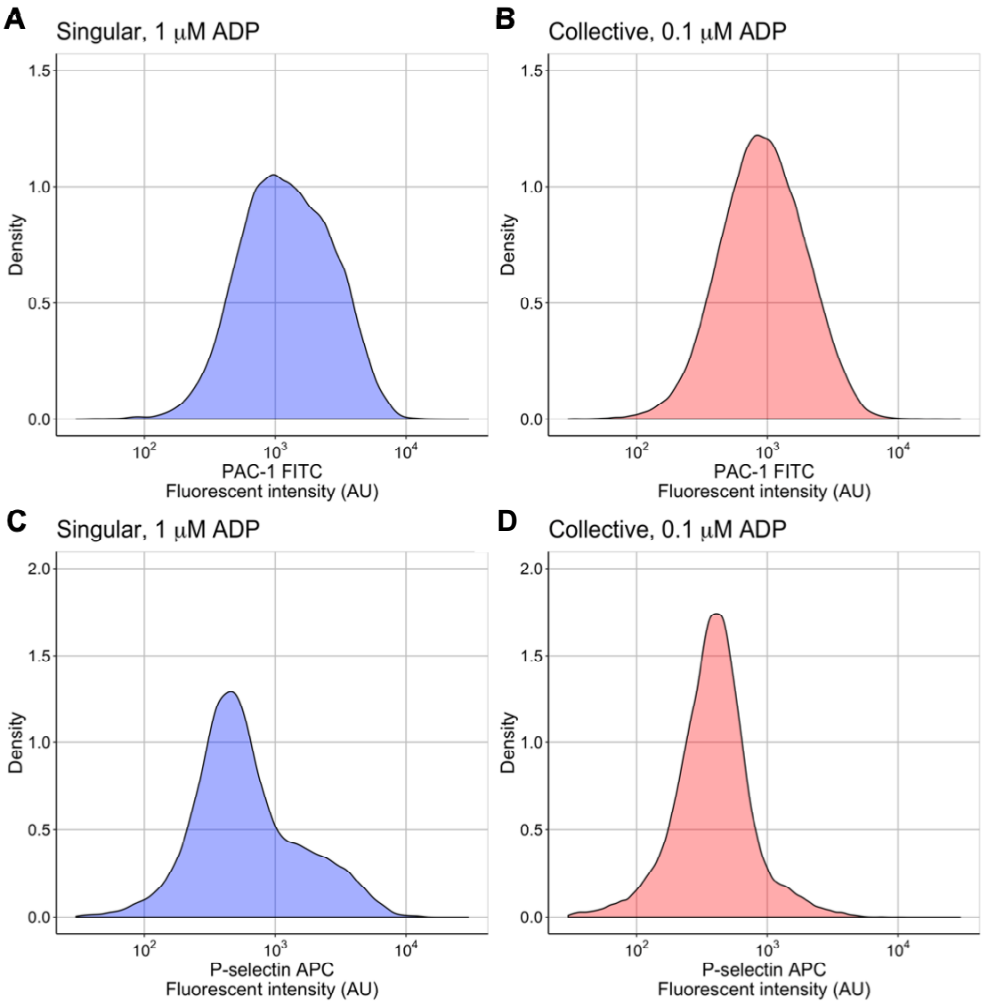


Figure B-18 Density plots of the intermediate platelet response to convulxin. Intermediate response shown for **(A and C)** single platelets to 1 μM ADP and **(B and D)** collective platelets to 0.1 μM ADP. Samples from donor 1 and same day. This type of data is further discussed in section 3.3.8.

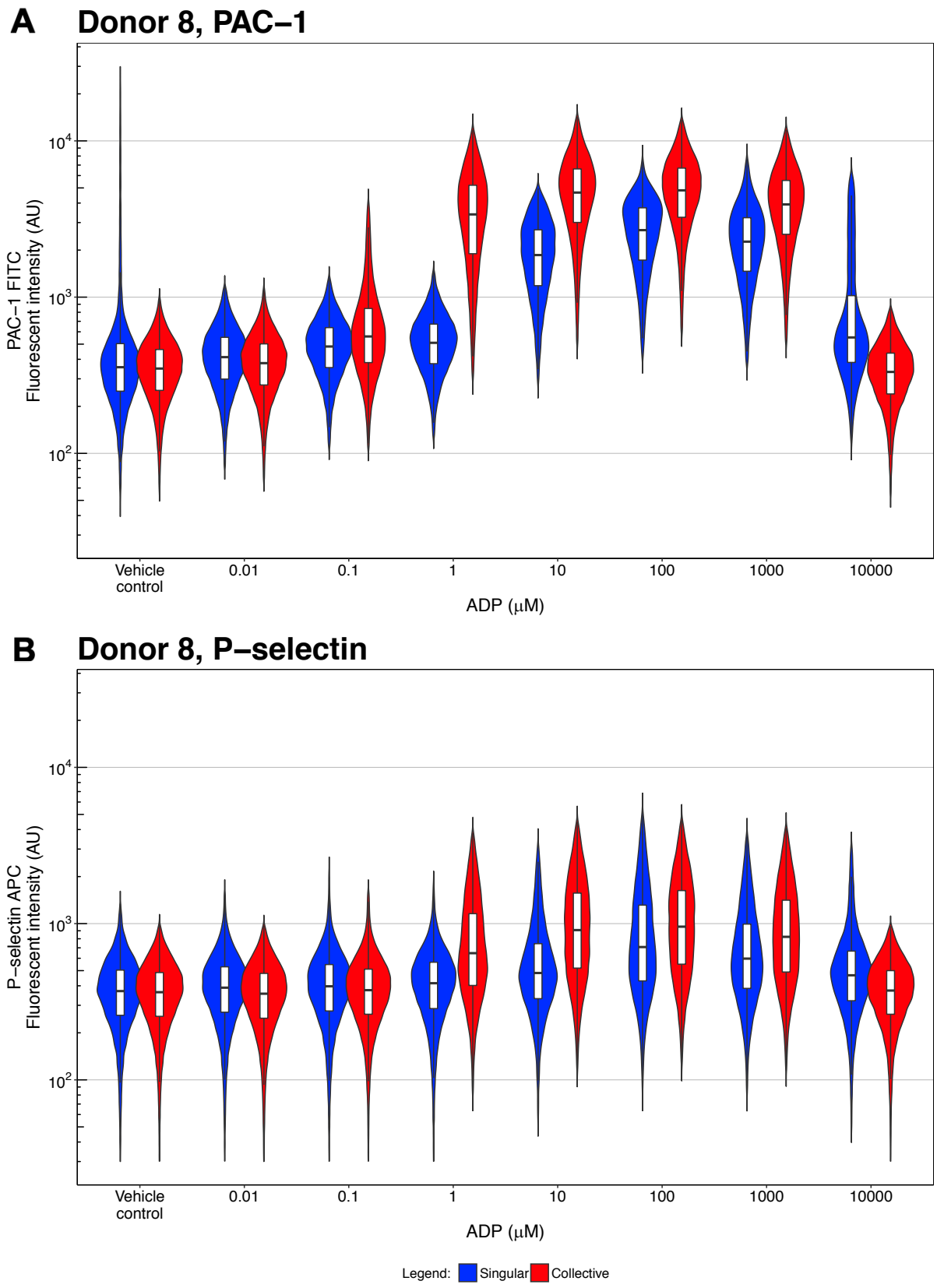


Figure B-19 Platelet responses of donor 8. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 or **B)** P-selectin antibody in response to ADP stimulation in droplets (singular, blue) or in suspension (collective, red). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.5. The violin plots are formed with a Gaussian kernel of data between the first and 99th percentile and tails are not trimmed. This type of data is further discussed in section 3.3.8. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table B-16. A fluorescent intensity >1000 AU was used to determine positive platelets (for both PAC-1 and P-selectin).

Table B-16 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to ADP. Platelet activity measured with PAC-1 antibody and P-selectin antibody, blood from donor 8. This experiment was discussed in section 3.3.8. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to ADP stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to ADP stimulation for collective platelets.

Singular vs collective response						
	PAC-1			P-selectin		
ADP (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9211	0.9171	0.9252	0.9869	0.9846	0.9891
0.01	0.9934	0.9915	0.9954	0.9810	0.9790	0.9830
0.1	1.1834	1.1772	1.1896	0.9937	0.9904	0.9970
1	8.6531	8.4356	8.8761	1.3985	1.3895	1.4074
10	4.7390	4.4953	4.9958	1.5602	1.5443	1.5762
100	3.0400	2.8270	3.2691	1.2434	1.2248	1.2623
1000	2.5963	2.4621	2.7378	1.2611	1.2477	1.2747
10000	0.7433	0.7384	0.7482	0.8981	0.8946	0.9017

Singular vehicle control vs convulxin response						
	PAC-1			P-selectin		
ADP (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	0.9326	0.9283	0.9369	1.0053	1.0024	1.0081
0.1	0.9506	0.9455	0.9557	1.0141	1.0105	1.0177
1	0.9656	0.9605	0.9706	1.0149	1.0117	1.0181
10	4.8043	4.6614	4.9516	1.1563	1.1488	1.1639
100	9.8783	9.3696	10.4146	1.5132	1.4942	1.5324
1000	7.3395	7.0821	7.6063	1.3087	1.2982	1.3193
10000	1.2318	1.2221	1.2415	1.0973	1.0924	1.1021

Collective vehicle control vs convulxin response						
	PAC-1			P-selectin		
ADP (μM)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0058	1.0044	1.0072	0.9993	0.9981	1.0005
0.1	1.2212	1.2159	1.2266	1.0211	1.0193	1.0229
1	9.0704	8.8439	9.3028	1.4381	1.4295	1.4468
10	24.7172	23.6646	25.8165	1.8280	1.8131	1.8431
100	32.6019	31.0112	34.2742	1.9066	1.8904	1.9230
1000	20.6875	19.8857	21.5216	1.6724	1.6601	1.6849
10000	0.9940	0.9930	0.9950	0.9986	0.9974	0.9998

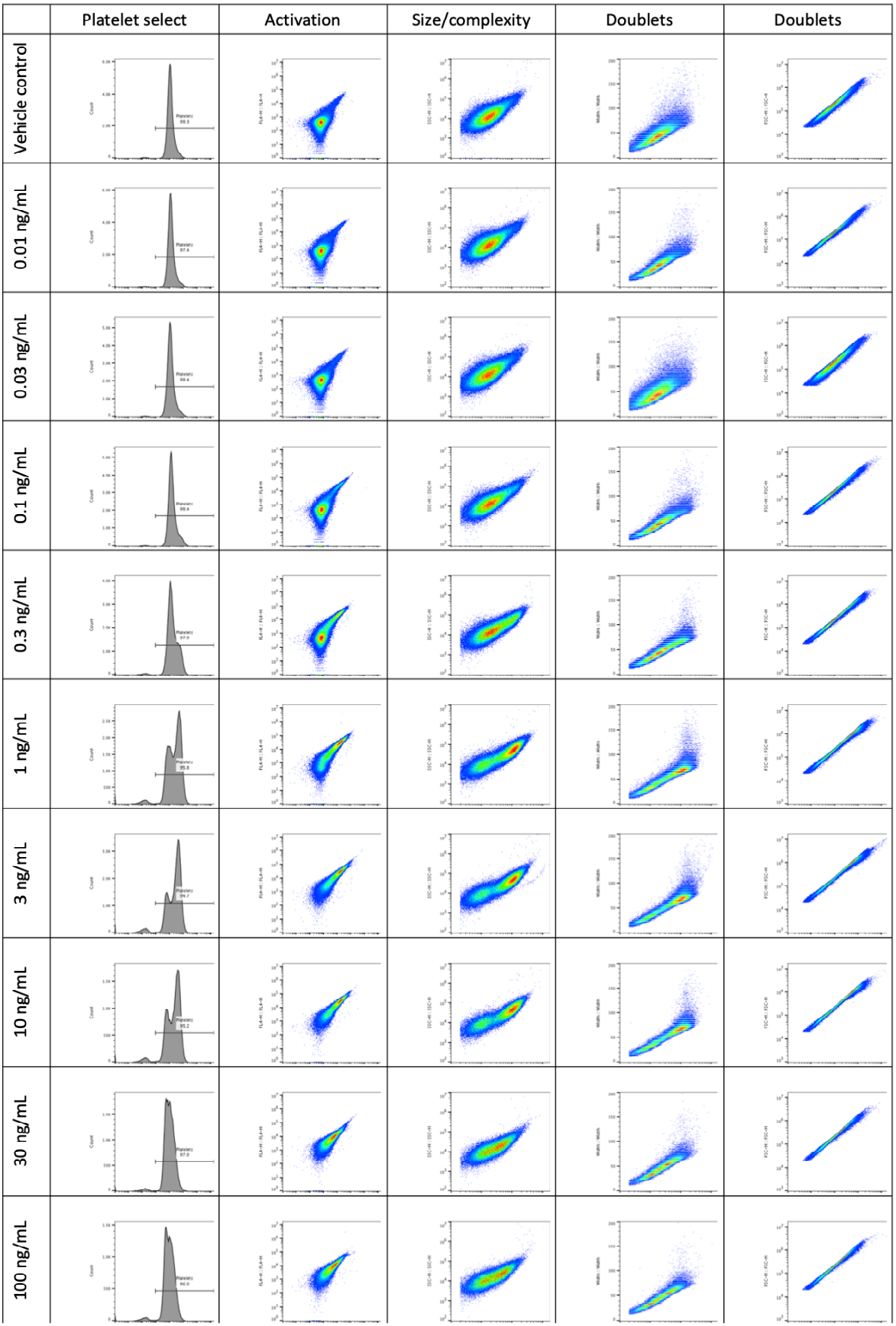


Figure B-20 Preliminary data of platelet aggregates in droplets. Platelets were added so that droplets contained 5-15 platelets. No PAC-1 or anti-CD61 was used that could potentially disrupt aggregation. CD63, CD42b and P-selectin antibodies were used instead. Columns represent: 1) CD42b (PE) histogram to select platelets, 2) Platelet activity measured with CD63 (FITC) and P-selectin (APC), 3) Platelet(aggregate) size represented as forward (indicator for size) and sideward (indicator of granularity) scatter, 4) Doublets/aggregates detection shown as forward scatter height to width 5) Doublets/aggregates detection shown as forward scatter area to height.

Appendix C

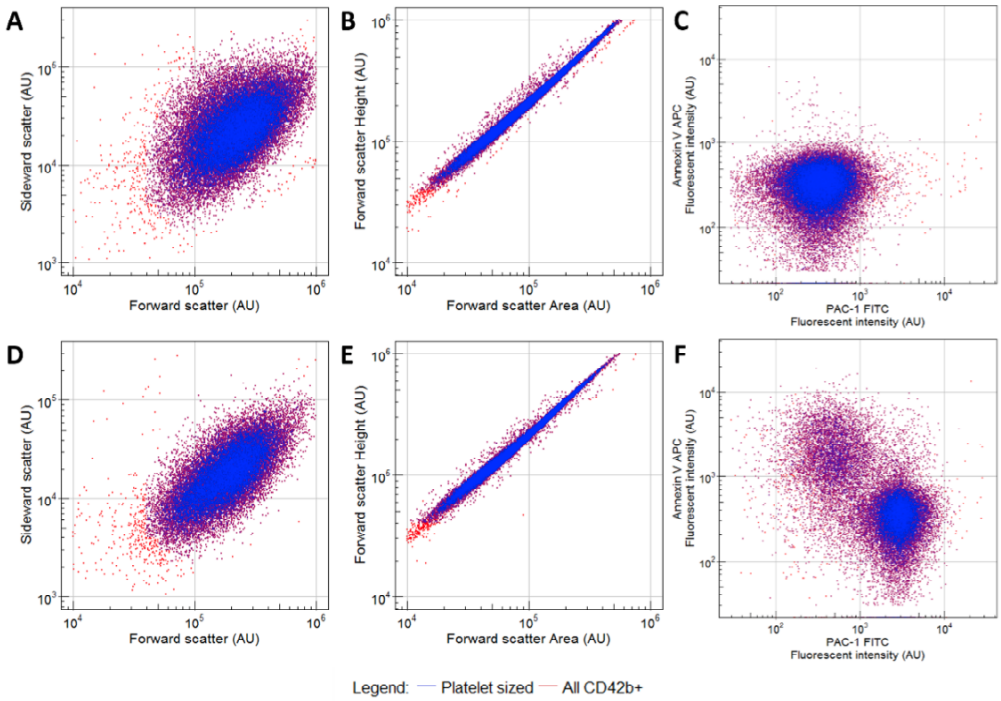


Figure C-1 Single platelet (in droplets) example of the effect of platelet gating strategy used in the presence of $CaCl_2$. Events within the platelet gate are shown in blue and all events positive for the platelet specific antibody CD42b are shown in red. **A)** Forward and sideward scatter gate. **B)** the doublet exclusion gate with the area vs height of forward scatter signal. **C)** the effect of the gate on the platelet activity of both PAC-1 and Annexin V signal. **D-F)** same for sample stimulated with 100 ng/mL of convulxin.

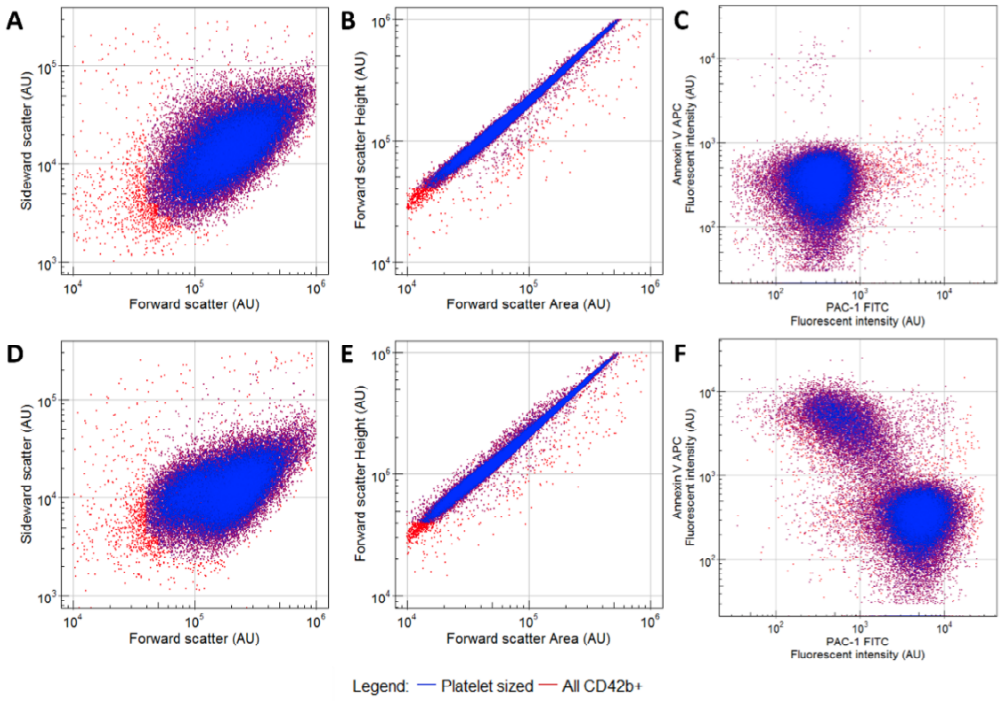


Figure C-2 Platelet collective (in suspension) example of the effect of platelet gating strategy used in the presence of $CaCl_2$. Events within the platelet gate are shown in blue and all events positive for the platelet specific antibody CD42b are shown in red. **A)** Forward and sideward scatter gate. **B)** the doublet exclusion gate with the area vs height of forward scatter signal. **C)** the effect of the gate on the platelet activity of both PAC-1 and Annexin V signal. **D-F)** same for sample stimulated with 100 ng/mL of convulxin.

Appendix C

Table C-1 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with PAC-1 antibody and Annexin V antibody, blood from donor 1. This experiment was discussed in section 4.3.1. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	PAC-1			Annexin V		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9850	0.9830	0.9871	0.9994	0.9986	1.0002
0.01	0.9995	0.9970	1.0020	1.0005	0.9996	1.0013
0.1	1.2932	1.2850	1.3014	1.0025	1.0014	1.0037
0.3	3.7593	3.7017	3.8179	1.0183	1.0166	1.0201
1	47.0846	43.8621	50.5439	0.9971	0.9955	0.9986
3	17.2605	16.4043	18.1614	1.0495	1.0466	1.0523
10	4.5724	4.3548	4.8009	1.0462	1.0420	1.0503
100	1.3759	1.3372	1.4157	1.0379	1.0297	1.0461

Singular vehicle control vs convulxin response						
	PAC-1			Annexin V		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0000	0.9973	1.0027	0.9992	0.9982	1.0001
0.1	1.0550	1.0513	1.0587	1.0023	1.0013	1.0034
0.3	1.0227	1.0195	1.0260	1.0033	1.0021	1.0045
1	1.3345	1.3248	1.3443	1.0089	1.0075	1.0104
3	1.8118	1.7914	1.8325	1.0108	1.0092	1.0124
10	4.7974	4.6742	4.9238	1.0433	1.0403	1.0464
100	3.9729	3.8894	4.0582	1.2570	1.2492	1.2648

Collective vehicle control vs convulxin response						
	PAC-1			Annexin V		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0147	1.0129	1.0165	1.0002	0.9995	1.0009
0.1	1.3851	1.3772	1.3930	1.0055	1.0045	1.0064
0.3	3.9031	3.8440	3.9632	1.0223	1.0208	1.0238
1	63.7899	59.4445	68.4529	1.0066	1.0056	1.0076
3	31.7479	30.2104	33.3638	1.0615	1.0590	1.0639
10	22.2689	21.3680	23.2078	1.0921	1.0891	1.0952
100	5.5494	5.4443	5.6566	1.3054	1.2989	1.3119

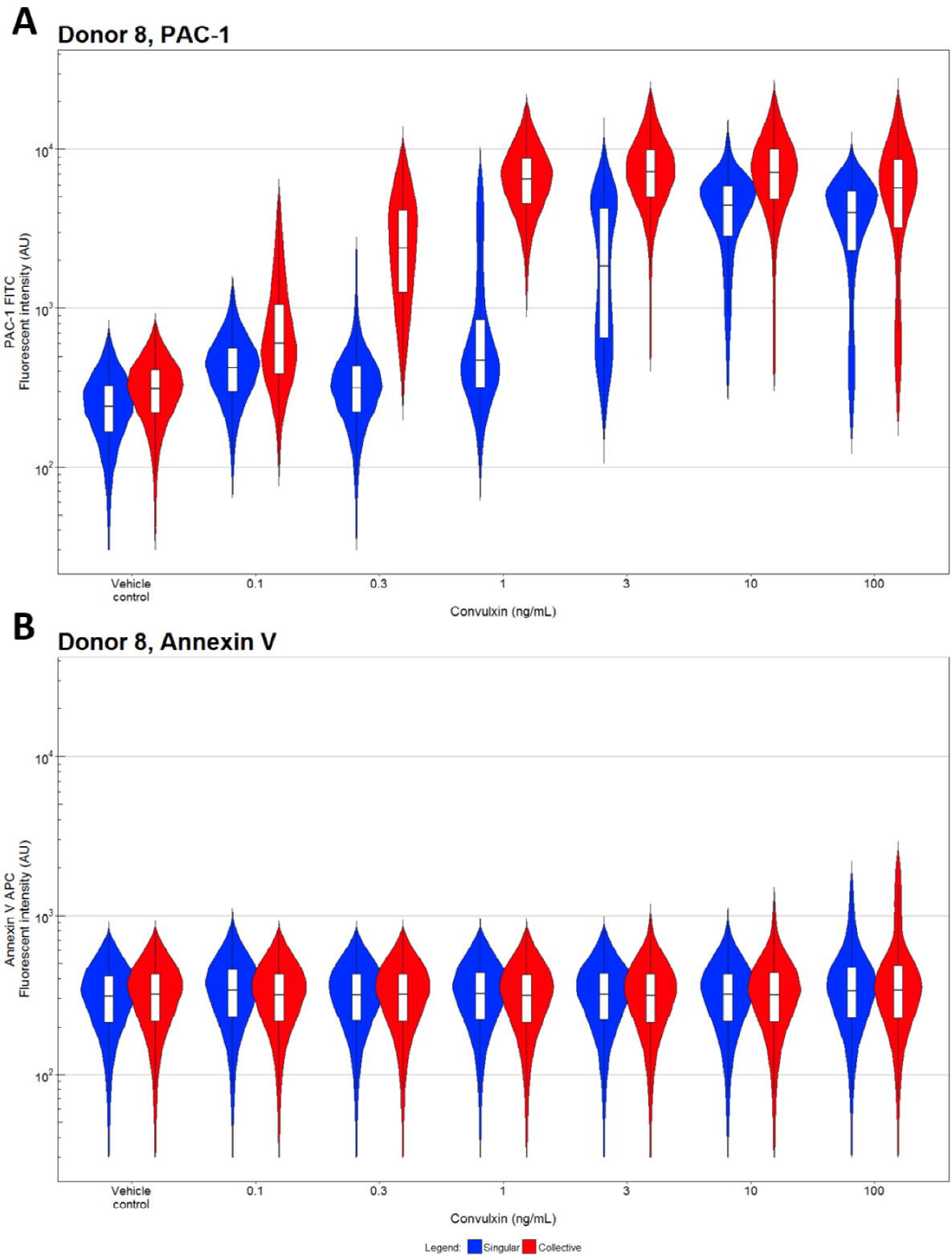


Figure C-3 Platelet responses of donor 8. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 antibody or **B)** Annexin V binding in response to convulxin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelet stimulation in the presence of $CaCl_2$ (2 mM) and Rivaroxaban (0.1 µM in 0.01% DMSO). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.6. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile, tails are not trimmed. N=2. Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-2. The RA for singular compared to collective for PAC-1 activation peaks at 1 ng/mL convulxin at 81. Singular RA vehicle compared to convulxin stimulation peaks at 10 ng/mL at 12, while collective RA peaks at 1 ng/mL at 104. The RA for Annexin V binding were all <2 including singular compared to collective and vehicle compared to convulxin. This experiment was discussed in section 4.3.1.

Table C-2 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to convulxin. Platelet activity measured with PAC-1 antibody and Annexin V antibody, blood from donor 8. This experiment was discussed in section 4.3.1. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to convulxin stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to convulxin stimulation for collective platelets.

Singular vs collective response						
	PAC-1			Annexin V		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	1.0011	1.0003	1.0019	1.0012	1.0006	1.0018
0.1	1.3310	1.3231	1.3389	0.9957	0.9945	0.9969
0.3	5.1696	5.0741	5.2670	1.0009	1.0002	1.0015
1	81.6911	74.5818	89.4779	1.0007	0.9999	1.0015
3	15.3406	14.4520	16.2839	1.0066	1.0054	1.0079
10	1.9719	1.8482	2.1039	1.0104	1.0087	1.0121
100	1.1397	1.0952	1.1861	1.0450	1.0408	1.0491

Singular vehicle control vs convulxin response						
	PAC-1			Annexin V		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.1	1.0311	1.0286	1.0337	1.0060	1.0048	1.0072
0.3	1.0312	1.0290	1.0333	1.0007	1.0001	1.0013
1	1.2846	1.2769	1.2924	1.0016	1.0009	1.0023
3	2.6929	2.6419	2.7449	1.0025	1.0016	1.0035
10	12.4633	11.8760	13.0796	1.0061	1.0048	1.0074
100	6.5249	6.3152	6.7415	1.0431	1.0400	1.0461

Collective vehicle control vs convulxin response						
	PAC-1			Annexin V		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.1	1.3709	1.3634	1.3784	1.0005	0.9999	1.0011
0.3	5.3249	5.2270	5.4247	1.0004	0.9998	1.0010
1	104.8229	95.7194	114.7922	1.0012	1.0005	1.0018
3	41.2644	38.9962	43.6644	1.0080	1.0070	1.0090
10	24.5493	23.5113	25.6331	1.0154	1.0141	1.0166
100	7.4282	7.2604	7.5999	1.0887	1.0857	1.0917

Table C-3 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to dual agonist stimulation with convulxin and thrombin. Platelet activity measured with PAC-1 antibody and Annexin V antibody, blood from donor 1. This experiment was discussed in section 4.3.3. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to dual agonist stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to dual agonist stimulation for collective platelets.

Singular vs collective response						
	PAC-1			Annexin V		
Convulxin (ng/mL) / Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	0.9960	0.9949	0.9971	0.9956	0.9946	0.9966
0.01 / 0.0001	1.0066	1.0055	1.0078	1.0004	0.9998	1.0010
0.03 / 0.0003	1.0509	1.0483	1.0535	0.9997	0.9988	1.0005
0.1 / 0.001	1.2871	1.2805	1.2937	0.9983	0.9974	0.9991
0.3 / 0.003	4.4001	4.3243	4.4772	1.0005	0.9996	1.0014
1 / 0.01	25.7816	24.6020	27.0178	1.0075	1.0050	1.0099
3 / 0.03	4.3847	4.2307	4.5443	1.0503	1.0461	1.0545
10 / 0.1	2.7115	2.6491	2.7754	1.1164	1.1095	1.1232
30 / 0.3	1.6083	1.5797	1.6374	1.4247	1.4131	1.4365
100 / 1	0.8474	0.8347	0.8604	1.0412	1.0269	1.0556
300 / 3	0.9007	0.8894	0.9121	1.0230	1.0024	1.0441

Singular vehicle control vs dual agonist stimulation response						
	PAC-1			Annexin V		
Convulxin (ng/mL) / Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01 / 0.0001	0.9970	0.9957	0.9982	0.9955	0.9945	0.9966
0.03 / 0.0003	1.0000	0.9984	1.0017	0.9964	0.9952	0.9977
0.1 / 0.001	1.0087	1.0069	1.0105	0.9980	0.9967	0.9992
0.3 / 0.003	1.0583	1.0545	1.0622	0.9971	0.9959	0.9984
1 / 0.01	1.0877	1.0828	1.0926	1.0133	1.0111	1.0155
3 / 0.03	2.7056	2.6512	2.7611	1.0310	1.0278	1.0342
10 / 0.1	1.9468	1.9196	1.9743	1.0895	1.0846	1.0945
30 / 0.3	1.7627	1.7398	1.7858	1.0907	1.0856	1.0959
100 / 1	2.1154	2.0882	2.1429	1.8040	1.7843	1.8240
300 / 3	1.6438	1.6257	1.6620	2.5429	2.5001	2.5864

Collective vehicle control vs dual agonist stimulation response						
	PAC-1			Annexin V		
Convulxin (ng/mL) / Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01 / 0.0001	1.0076	1.0067	1.0086	1.0004	0.9999	1.0008
0.03 / 0.0003	1.0552	1.0529	1.0574	1.0005	1.0000	1.0010
0.1 / 0.001	1.3036	1.2972	1.3100	1.0006	1.0001	1.0011
0.3 / 0.003	4.6757	4.5967	4.7560	1.0020	1.0014	1.0026
1 / 0.01	28.1562	26.8733	29.5003	1.0254	1.0239	1.0269
3 / 0.03	11.9111	11.5654	12.2672	1.0876	1.0847	1.0905
10 / 0.1	5.3001	5.2024	5.3997	1.2217	1.2165	1.2269
30 / 0.3	2.8463	2.8114	2.8818	1.5609	1.5503	1.5716
100 / 1	1.7999	1.7855	1.8144	1.8866	1.8707	1.9026
300 / 3	1.4865	1.4772	1.4958	2.6130	2.5834	2.6429

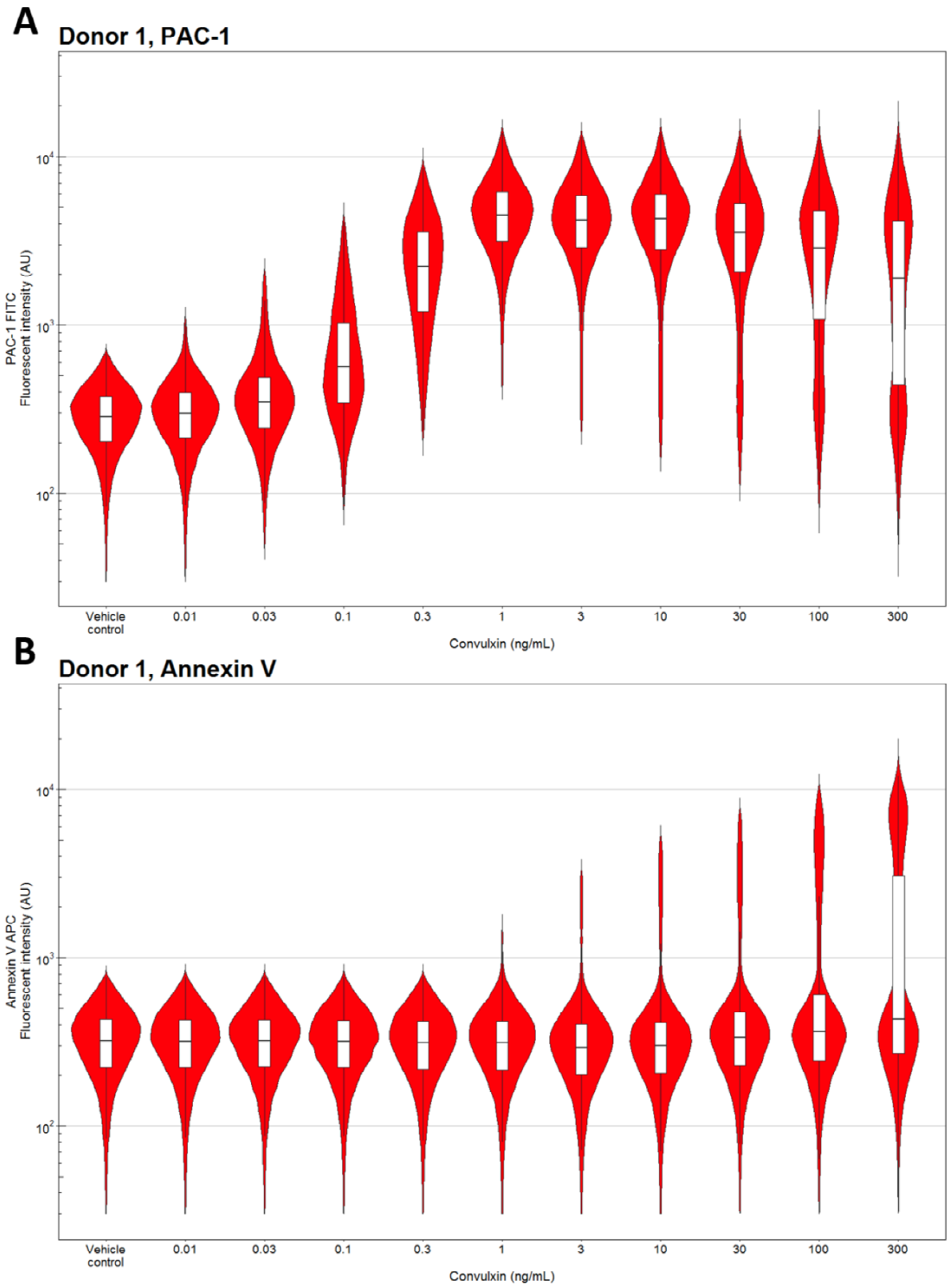


Figure C-4 Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 antibody or **B)** Annexin V binding in response to convulxin stimulation in suspension (collective). Platelet stimulation in the presence of $CaCl_2$ (2 mM), GPRP (20 mM) and Rivaroxaban (0.1 µM in 0.01% DMSO), blood from donor 1. Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.6. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile, tails are not trimmed. This experiment was discussed in section 4.3.3. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-4.

Table C-4 Relative activation and confidence intervals of dose response relationship of collective platelet activity in response to convulxin stimulation. Platelet activity measured with PAC-1 antibody and Annexin V antibody, blood from donor 1. This experiment was discussed in section 4.3.3. Top table shows the relative activation of dual agonist stimulation (addition of thrombin) compared to stimulation with convulxin alone. Bottom table shows the relative activation of convulxin stimulation compared to vehicle control.

Collective convulxin vs dual agonist stimulation response						
	PAC-1			Annexin V		
Convulxin (ng/mL) / Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01 / 0.0001	0.9977	0.9961	0.9993	1.0000	0.9994	1.0006
0.03 / 0.0003	1.0003	0.9967	1.0041	1.0000	0.9993	1.0006
0.1 / 0.001	0.9619	0.9533	0.9706	1.0003	0.9997	1.0010
0.3 / 0.003	0.9384	0.9102	0.9675	1.0012	1.0004	1.0019
1 / 0.01	0.8133	0.7455	0.8873	1.0126	1.0105	1.0147
3 / 0.03	0.6778	0.6389	0.7191	1.0419	1.0379	1.0458
10 / 0.1	0.4390	0.4193	0.4596	1.1348	1.1285	1.1410
30 / 0.3	0.3948	0.3816	0.4085	1.3894	1.3781	1.4008
100 / 1	0.4419	0.4316	0.4524	1.5076	1.4917	1.5236
300 / 3	0.5638	0.5541	0.5736	1.7202	1.6954	1.7454

Collective vehicle control vs convulxin response						
	PAC-1			Annexin V		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0100	1.0085	1.0114	1.0004	0.9998	1.0010
0.03	1.0548	1.0516	1.0580	1.0006	0.9999	1.0012
0.1	1.3552	1.3450	1.3655	1.0003	0.9997	1.0009
0.3	4.9825	4.8580	5.1103	1.0009	1.0002	1.0015
1	34.6207	32.1663	37.2624	1.0127	1.0111	1.0142
3	17.5731	16.6957	18.4968	1.0439	1.0411	1.0467
10	12.0730	11.5777	12.5895	1.0766	1.0728	1.0804
30	7.2097	6.9848	7.4417	1.1234	1.1184	1.1285
100	4.0732	3.9841	4.1643	1.2514	1.2435	1.2594
300	2.6367	2.5947	2.6794	1.5190	1.5053	1.5328

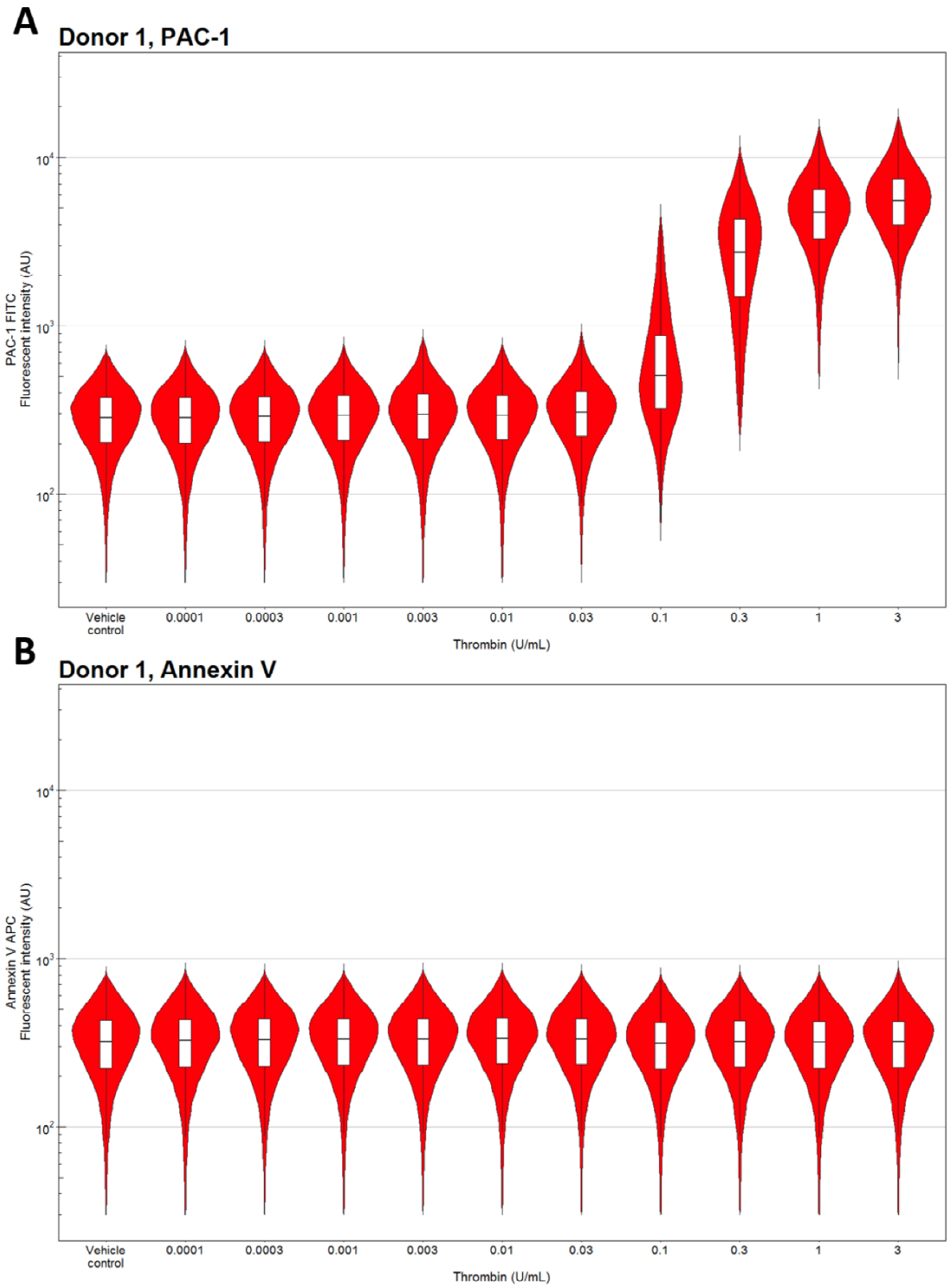


Figure C-5 Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 antibody or **B)** Annexin V binding in response to thrombin stimulation in suspension (collective). Platelet stimulation in the presence of $CaCl_2$ (2 mM), GPRP (20 mM) and Rivaroxaban (0.1 µM in 0.01% DMSO), blood from donor 1. Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.6. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile, tails are not trimmed. This experiment was discussed in section 4.3.3. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-5.

Table C-5 Relative activation and confidence intervals of dose response relationship of collective platelet activity in response to thrombin stimulation. Platelet activity measured with PAC-1 antibody and Annexin V antibody, blood from donor 1. This experiment was discussed in section 4.3.3. Top table shows the relative activation of dual agonist stimulation (addition of convulxin) compared to stimulation with thrombin alone. Bottom table shows the relative activation of convulxin stimulation compared to vehicle control.

Collective thrombin vs dual agonist stimulation response						
	PAC-1			Annexin V		
Convulxin (ng/mL) / Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01 / 0.0001	1.0072	1.0061	1.0083	0.9996	0.9990	1.0003
0.03 / 0.0003	1.0547	1.0524	1.0571	0.9999	0.9992	1.0005
0.1 / 0.001	1.3020	1.2956	1.3085	1.0003	0.9997	1.0009
0.3 / 0.003	4.6588	4.5801	4.7389	1.0014	1.0007	1.0022
1 / 0.01	28.1410	26.8588	29.4845	1.0247	1.0230	1.0263
3 / 0.03	11.8536	11.5093	12.2082	1.0869	1.0839	1.0898
10 / 0.1	4.1699	4.0885	4.2530	1.2215	1.2163	1.2268
30 / 0.3	0.4476	0.4336	0.4620	1.5592	1.5486	1.5699
100 / 1	0.0458	0.0424	0.0495	1.8819	1.8660	1.8980
300 / 3	0.0243	0.0220	0.0268	2.5992	2.5698	2.6291

Collective vehicle control vs thrombin response						
	PAC-1			Annexin V		
Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.0001	1.0005	0.9997	1.0012	1.0007	1.0001	1.0014
0.0003	1.0004	0.9997	1.0012	1.0007	1.0000	1.0013
0.001	1.0012	1.0004	1.0020	1.0004	0.9998	1.0009
0.003	1.0036	1.0026	1.0046	1.0006	1.0000	1.0013
0.01	1.0005	0.9998	1.0013	1.0007	1.0001	1.0014
0.03	1.0049	1.0037	1.0060	1.0007	1.0000	1.0013
0.1	1.2710	1.2627	1.2794	1.0001	0.9995	1.0007
0.3	6.3588	6.1758	6.5474	1.0011	1.0004	1.0017
1	39.2796	36.3428	42.4537	1.0025	1.0017	1.0033
3	61.2413	55.5417	67.5257	1.0053	1.0042	1.0064

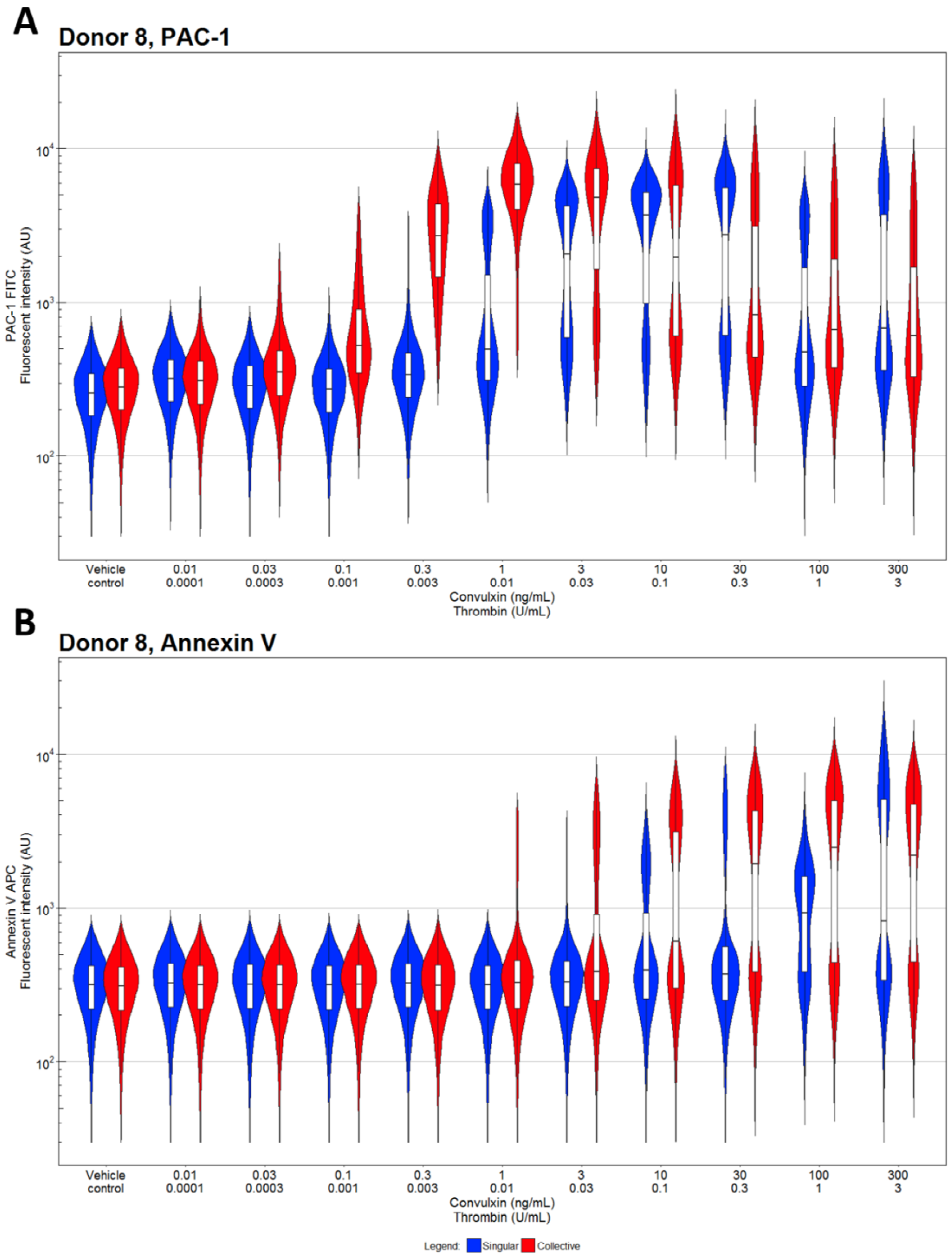


Figure C-6 Platelet responses of donor 8. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 antibody or **B)** Annexin V binding in response to convulxin and thrombin stimulation in droplets (singular, blue) or in suspension (collective, red). Platelet stimulation in the presence of $CaCl_2$ (2 mM), GPRP (20 mM) and Rivaroxaban (0.1 µM in 0.01% DMSO). Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.6. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile, tails are not trimmed. This experiment was discussed in section 4.3.3. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-6.

Table C-6 Relative activation and confidence intervals of dose response relationship of single and collective platelet activity in response to dual agonist stimulation with convulxin and thrombin. Platelet activity measured with PAC-1 antibody and Annexin V antibody, blood from donor 8. This experiment was discussed in section 4.3.3. Top table shows the relative activation (RA) of singular compared to collective response, middle table shows the RA of vehicle control compared to dual agonist stimulation for singular platelets and the bottom table shows the RA of vehicle control compared to dual agonist stimulation for collective platelets.

Singular vs collective response						
	PAC-1			Annexin V		
Convulxin (ng/mL) / Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
Vehicle control	1.0017	1.0009	1.0026	0.9997	0.9990	1.0004
0.01 / 0.0001	1.0069	1.0056	1.0082	0.9997	0.9988	1.0006
0.03 / 0.0003	1.0451	1.0429	1.0473	0.9993	0.9983	1.0003
0.1 / 0.001	1.2725	1.2663	1.2788	1.0000	0.9991	1.0010
0.3 / 0.003	6.2470	6.1163	6.3806	1.0018	1.0007	1.0028
1 / 0.01	14.1251	13.5744	14.6982	1.0696	1.0667	1.0724
3 / 0.03	1.9850	1.9346	2.0368	1.2818	1.2743	1.2894
10 / 0.1	0.6472	0.6327	0.6621	1.3735	1.3591	1.3880
30 / 0.3	0.6794	0.6673	0.6917	2.0126	1.9888	2.0368
100 / 1	1.1002	1.0847	1.1159	1.4128	1.3837	1.4424
300 / 3	0.9025	0.8901	0.9150	1.3445	1.3205	1.3689

Singular vehicle control vs dual agonist stimulation response						
	PAC-1			Annexin V		
Convulxin (ng/mL) / Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01 / 0.0001	1.0025	1.0015	1.0035	1.0015	1.0006	1.0024
0.03 / 0.0003	1.0008	0.9998	1.0018	1.0016	1.0006	1.0027
0.1 / 0.001	1.0077	1.0062	1.0093	1.0007	0.9998	1.0017
0.3 / 0.003	1.0412	1.0384	1.0439	1.0018	1.0008	1.0028
1 / 0.01	1.4206	1.4094	1.4320	1.0039	1.0028	1.0050
3 / 0.03	2.5555	2.5091	2.6027	1.0353	1.0323	1.0383
10 / 0.1	3.8827	3.8066	3.9602	1.3257	1.3169	1.3346
30 / 0.3	2.6829	2.6403	2.7261	1.1918	1.1853	1.1983
100 / 1	1.4627	1.4448	1.4808	1.9063	1.8738	1.9393
300 / 3	1.7227	1.7019	1.7438	1.9585	1.9312	1.9860

Collective vehicle control vs dual agonist stimulation response						
	PAC-1			Annexin V		
Convulxin (ng/mL) / Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01 / 0.0001	1.0076	1.0065	1.0088	1.0014	1.0008	1.0021
0.03 / 0.0003	1.0442	1.0420	1.0463	1.0012	1.0006	1.0019
0.1 / 0.001	1.2801	1.2741	1.2863	1.0011	1.0004	1.0017
0.3 / 0.003	6.4928	6.3579	6.6306	1.0039	1.0031	1.0047
1 / 0.01	20.0321	19.2662	20.8284	1.0740	1.0714	1.0767
3 / 0.03	5.0638	4.9729	5.1563	1.3274	1.3206	1.3343
10 / 0.1	2.5086	2.4808	2.5366	1.8214	1.8065	1.8363
30 / 0.3	1.8195	1.8047	1.8345	2.3994	2.3739	2.4251
100 / 1	1.6064	1.5952	1.6178	2.6939	2.6626	2.7255
300 / 3	1.5520	1.5417	1.5623	2.6339	2.6041	2.6639

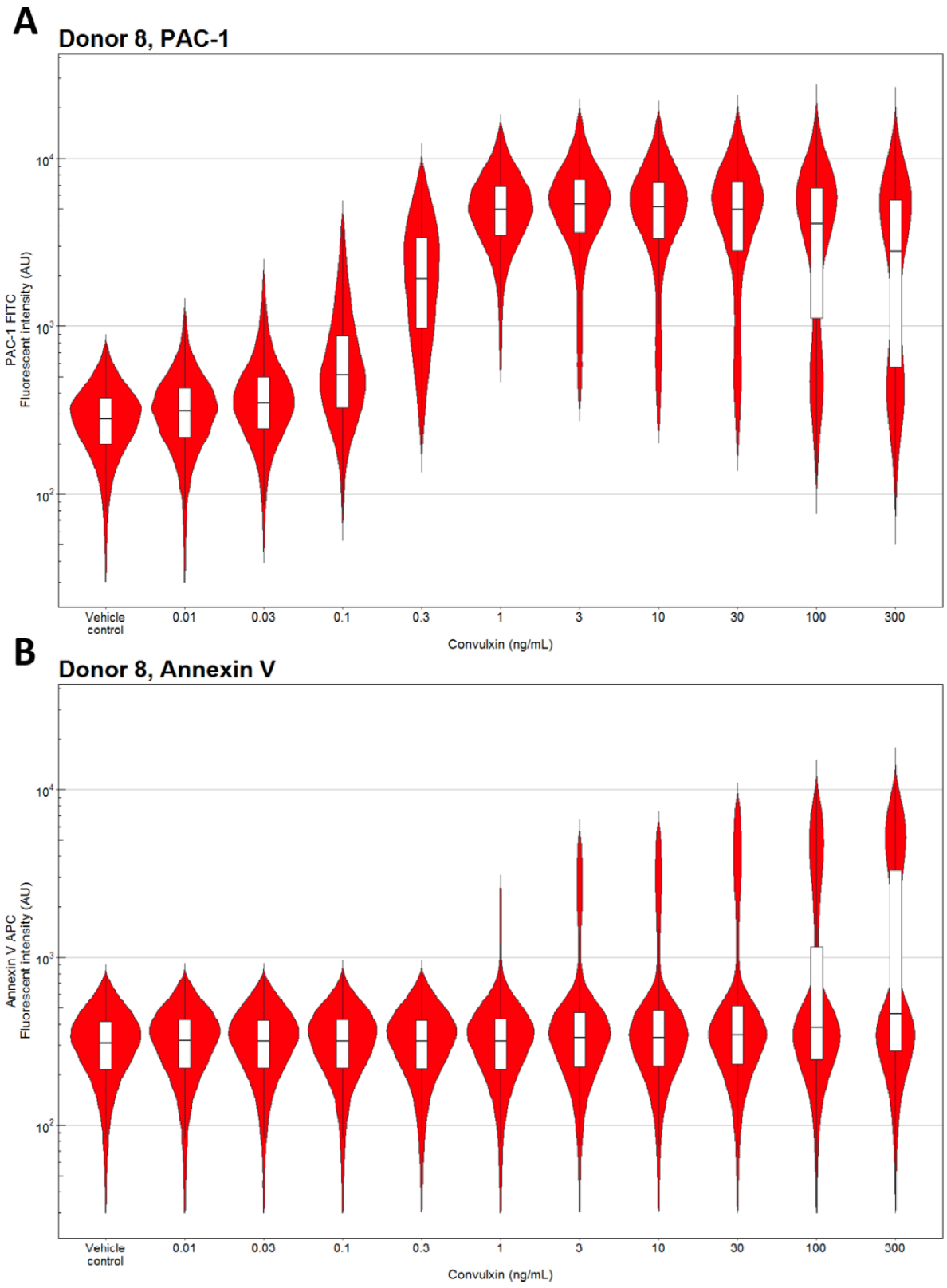


Figure C-7 Platelet responses of donor 8. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 antibody or **B)** Annexin V binding in response to convulxin stimulation in suspension (collective). Platelet stimulation in the presence of $CaCl_2$ (2 mM), GPRP (20 mM) and Rivaroxaban (0.1 µM in 0.01% DMSO), blood from donor 8. Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.6. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile, tails are not trimmed. This experiment was discussed in section 4.3.3. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-7.

Table C-7 Relative activation and confidence intervals of dose response relationship of collective platelet activity in response to convulxin stimulation. Platelet activity measured with PAC-1 antibody and Annexin V antibody, blood from donor 8. This experiment was discussed in section 4.3.3. Top table shows the relative activation of dual agonist stimulation (addition of thrombin) compared to stimulation with convulxin alone. Bottom table shows the relative activation of convulxin stimulation compared to vehicle control.

Collective convulxin vs dual agonist stimulation response						
	PAC-1			Annexin V		
Convulxin (ng/mL) / Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01 / 0.0001	0.9953	0.9934	0.9973	1.0012	1.0004	1.0020
0.03 / 0.0003	0.9959	0.9925	0.9994	1.0010	1.0002	1.0018
0.1 / 0.001	1.0134	1.0052	1.0216	0.9989	0.9979	0.9999
0.3 / 0.003	1.7108	1.6606	1.7624	1.0012	1.0001	1.0023
1 / 0.01	0.4950	0.4530	0.5409	1.0492	1.0458	1.0525
3 / 0.03	0.3334	0.3166	0.3510	1.2131	1.2053	1.2210
10 / 0.1	0.2278	0.2185	0.2375	1.6224	1.6073	1.6376
30 / 0.3	0.2375	0.2297	0.2456	2.0488	2.0246	2.0732
100 / 1	0.3940	0.3850	0.4033	1.9987	1.9712	2.0265
300 / 3	0.5435	0.5336	0.5536	1.6445	1.6201	1.6693

Collective vehicle control vs convulxin response						
	PAC-1			Annexin V		
Convulxin (ng/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01	1.0123	1.0106	1.0141	1.0002	0.9995	1.0010
0.03	1.0484	1.0453	1.0515	1.0003	0.9995	1.0010
0.1	1.2633	1.2550	1.2715	1.0021	1.0012	1.0031
0.3	3.7953	3.7162	3.8761	1.0026	1.0017	1.0036
1	40.4688	37.3739	43.8200	1.0237	1.0216	1.0258
3	15.1889	14.4723	15.9410	1.0942	1.0899	1.0986
10	11.0127	10.5798	11.4634	1.1227	1.1176	1.1277
30	7.6612	7.4155	7.9150	1.1711	1.1650	1.1773
100	4.0769	3.9877	4.1681	1.3478	1.3378	1.3579
300	2.8554	2.8069	2.9047	1.6016	1.5860	1.6173

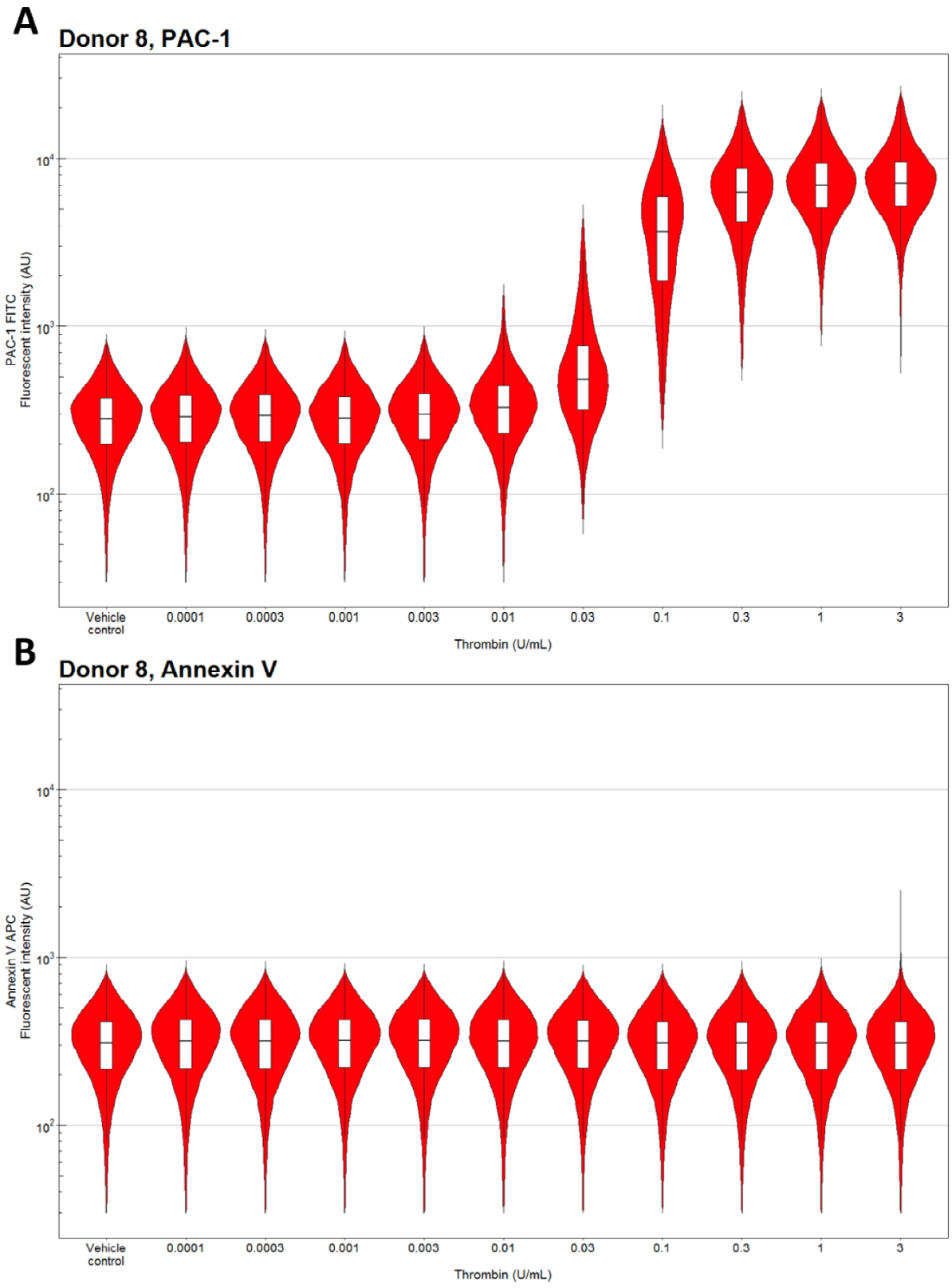
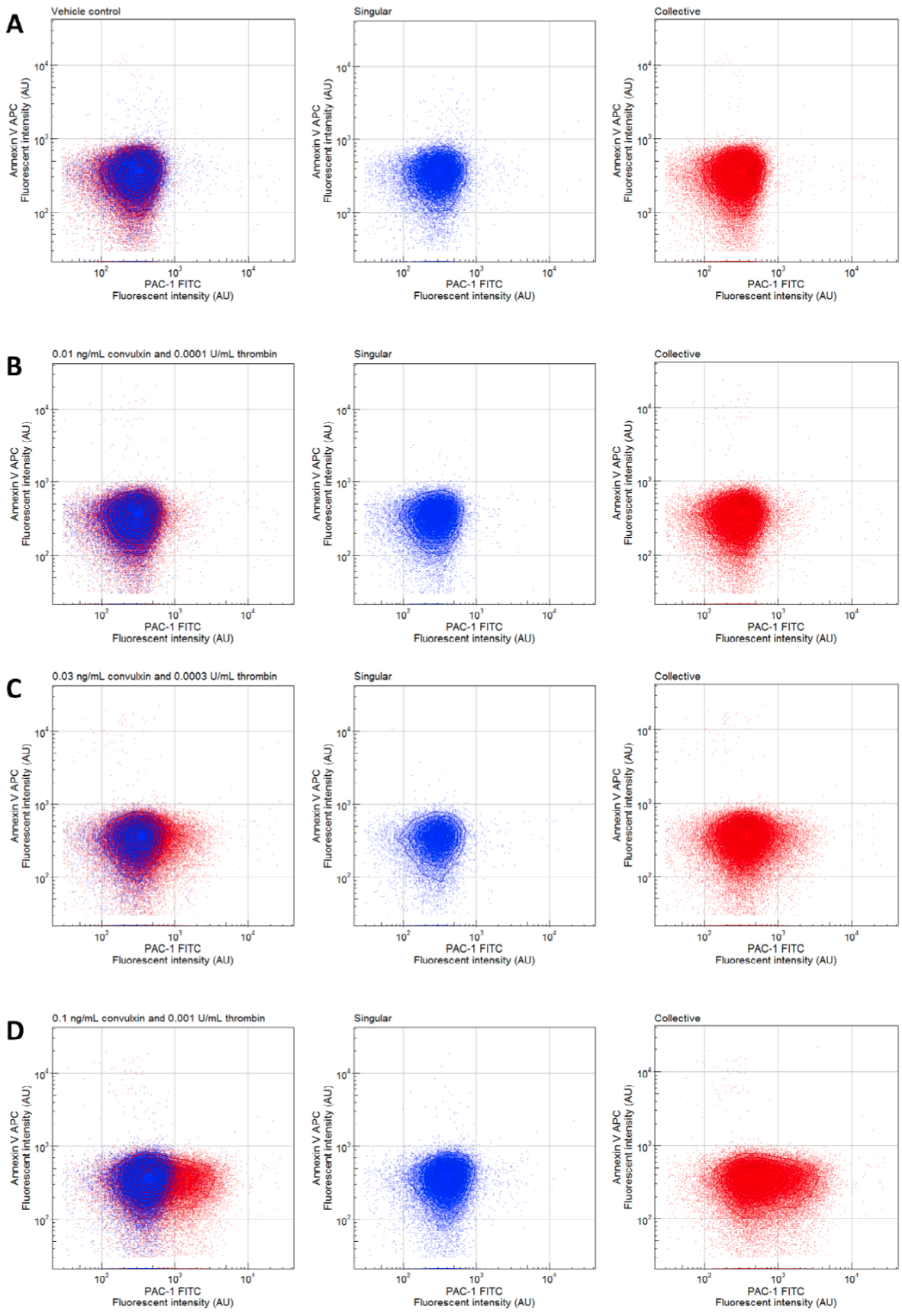


Figure C-8 Platelet responses of donor 8. Violin and box plots of the fluorescent intensity observed with **A)** PAC-1 antibody or **B)** Annexin V binding in response to thrombin stimulation in suspension (collective). Platelet stimulation in the presence of $CaCl_2$ (2 mM), GPRP (20 mM) and Rivaroxaban (0.1 µM in 0.01% DMSO), blood from donor 8. Platelets were selected with anti-CD42b and following the gating procedure described in section 2.3.6. Violin plots are formed with a Gaussian kernel of data between the first and 99th percentile, tails are not trimmed. This experiment was discussed in section 4.3.3. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-8.

Table C-8 Relative activation and confidence intervals of dose response relationship of collective platelet activity in response to thrombin stimulation. Platelet activity measured with PAC-1 antibody and Annexin V antibody, blood from donor 8. This experiment was discussed in section 4.3.3. Top table shows the relative activation of dual agonist stimulation (addition of convulxin) compared to stimulation with thrombin alone. Bottom table shows the relative activation of thrombin stimulation compared to vehicle control.

Collective thrombin vs dual agonist stimulation response						
	PAC-1			Annexin V		
Convulxin (ng/mL) / Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.01 / 0.0001	1.0071	1.0058	1.0085	1.0005	0.9996	1.0014
0.03 / 0.0003	1.0440	1.0417	1.0462	1.0010	1.0002	1.0018
0.1 / 0.001	1.2799	1.2737	1.2860	1.0005	0.9997	1.0013
0.3 / 0.003	6.4892	6.3543	6.6270	1.0039	1.0030	1.0048
1 / 0.01	19.6842	18.9309	20.4675	1.0724	1.0697	1.0752
3 / 0.03	4.2379	4.1583	4.3189	1.3279	1.3211	1.3348
10 / 0.1	0.2992	0.2886	0.3101	1.8201	1.8053	1.8351
30 / 0.3	0.0438	0.0404	0.0476	2.3933	2.3679	2.4190
100 / 1	0.0182	0.0162	0.0205	2.6790	2.6478	2.7105
300 / 3	0.0210	0.0188	0.0234	2.6066	2.5769	2.6365

Collective vehicle control vs thrombin response						
	PAC-1			Annexin V		
Thrombin (U/mL)	Relative activation	Confidence interval		Relative activation	Confidence interval	
0.0001	1.0005	0.9994	1.0016	1.0009	1.0001	1.0017
0.0003	1.0002	0.9991	1.0012	1.0002	0.9995	1.0009
0.001	1.0002	0.9992	1.0013	1.0006	0.9998	1.0013
0.003	1.0006	0.9995	1.0016	1.0000	0.9993	1.0007
0.01	1.0177	1.0157	1.0197	1.0015	1.0006	1.0023
0.03	1.1949	1.1882	1.2017	0.9996	0.9990	1.0003
0.1	8.3856	8.1028	8.6782	1.0007	0.9999	1.0015
0.3	41.5039	38.2733	45.0072	1.0025	1.0016	1.0035
1	88.0957	78.3278	99.0819	1.0056	1.0044	1.0067
3	73.9146	66.3487	82.3433	1.0105	1.0090	1.0120



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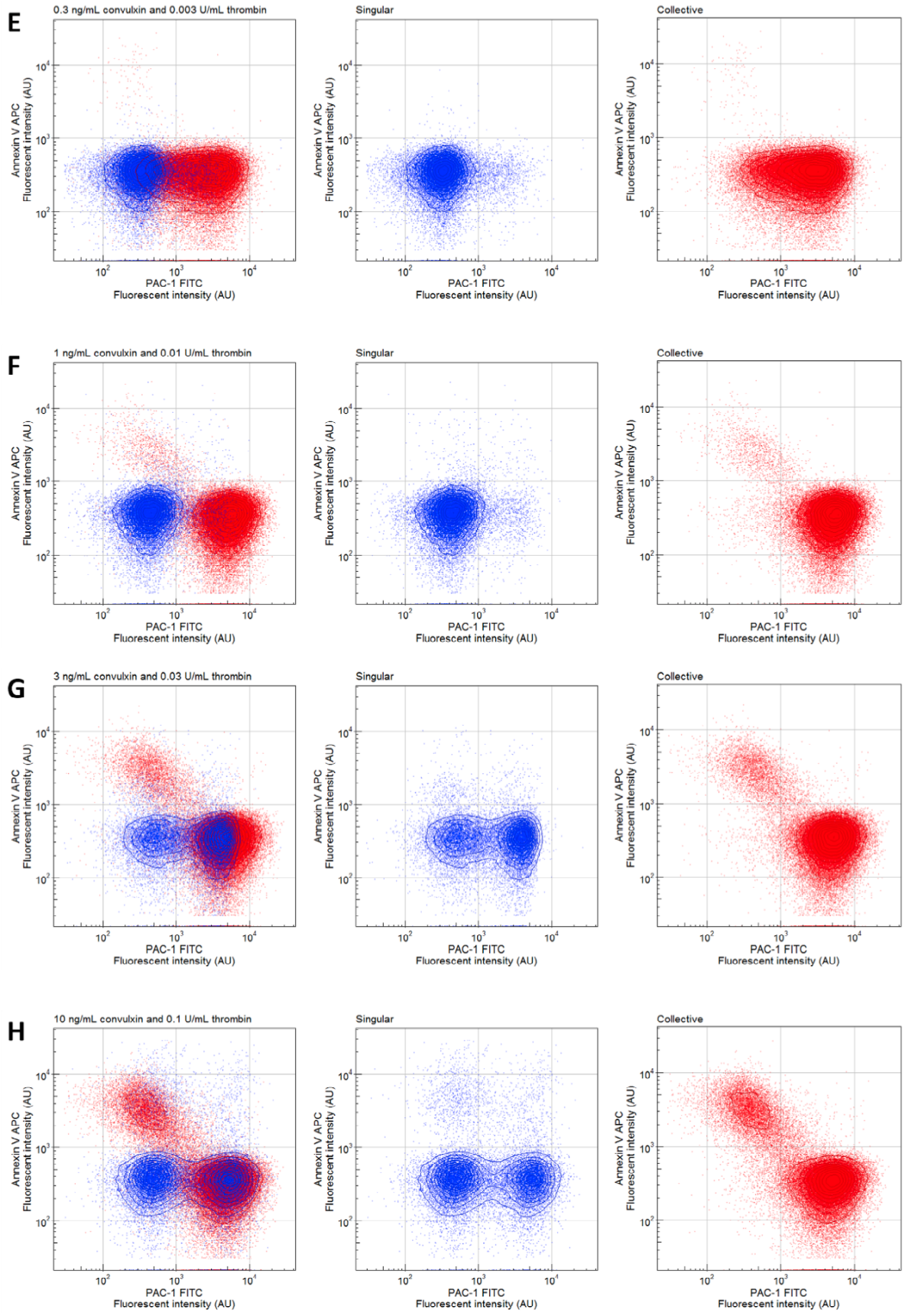

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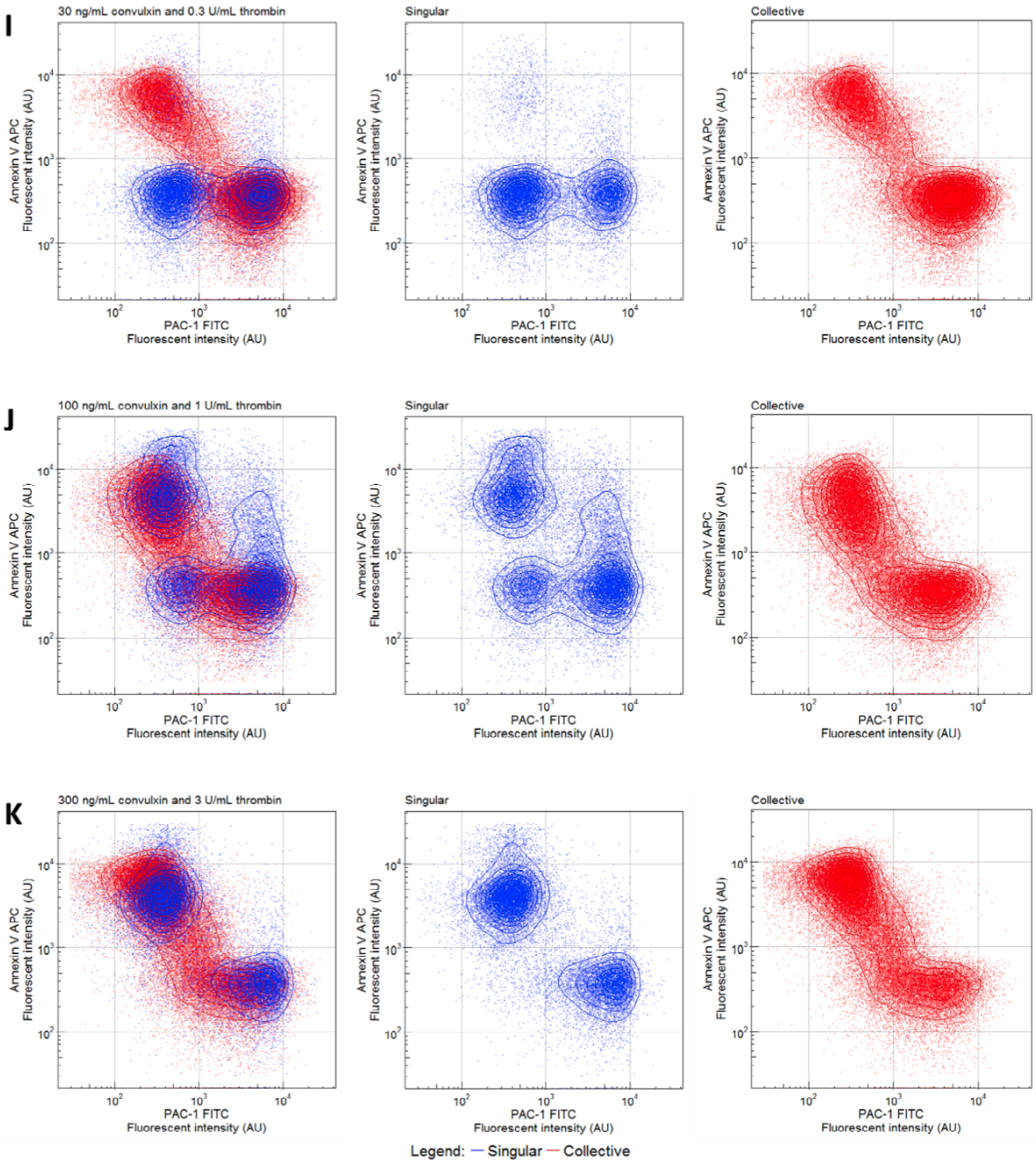

Figure C-9 Relationship between PAC-1 and Annexin V binding of singular and collective platelets. Platelets stimulated with **A)** vehicle control, **B)** 0.01 ng/mL of convulxin and 0.0001 U/mL of thrombin, **C)** 0.03 ng/mL of convulxin and 0.0003 U/mL of thrombin, **D)** 0.1 ng/mL of convulxin and 0.001 U/mL of thrombin, **E)** 0.3 ng/mL of convulxin and 0.003 U/mL of thrombin, **F)** 1 ng/mL of convulxin and 0.01 U/mL of thrombin, **G)** 3 ng/mL of convulxin and 0.03 U/mL of thrombin, **H)** 10 ng/mL of convulxin and 0.1 U/mL of thrombin, **I)** 30 ng/mL of convulxin and 0.3 U/mL of thrombin, **J)** 100 ng/mL of convulxin and 1 U/mL of thrombin and **K)** 300 ng/mL of convulxin and 3 U/mL of thrombin. Each row consists of 3 scatter plots of respectively an overlay of singular and collective platelet response, only singular and only collective platelet response. Data is selected from the experiment depicted in Figure 4-4. Data is shown as scatter plot with contour plot showing densities.

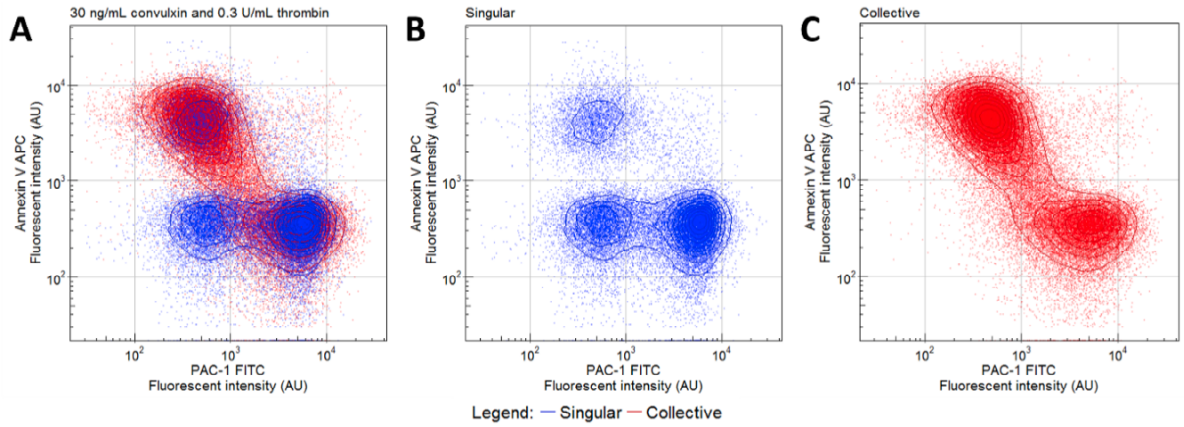

Figure C-10 Platelet responses of donor 8. Relationship between PAC-1 and Annexin V binding of singular and collective platelets stimulated with 30 ng/mL of convulxin and 0.3 U/mL of thrombin. Scatter plot of **A)** overlay of singular and collective platelet response, **B)** only singular and **C)** only collective platelet response. Data is selected from the experiment depicted in Figure C-6. Data is shown as scatter plot with contour plot showing densities. The procoagulant population consists of 16% of singlular platelets and 58% of collective platelets. The proaggregatory population consists of 59% of singlular platelets while 25% of singular platelets is non-active.

Table C-9 Relative activation and confidence intervals of singular platelet activity compared to collective in response to dual agonist stimulation with 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Platelet activity measured with different panels of antibodies, blood from donor 1. This experiment was discussed in section 4.3.5. Antibody panels used were panel A: PAC-1 (FITC) and P-selectin (APC), panel B: PAC-1 (FITC) and Annexin V (APC), panel C: P-selectin (FITC) and Annexin V (APC) and panel D: CD63 (FITC) and P-selectin (APC).

Singular vs collective response						
	Antibody 1 (FITC)			Antibody 2 (APC)		
Antibody panel	Relative activation	Confidence interval		Relative activation	Confidence interval	
A	0.4272	0.4140	0.4409	3.3621	3.2067	3.5251
B	0.9753	0.9594	0.9915	1.2191	1.2025	1.2359
C	1.7584	1.6035	1.9282	1.4087	1.3866	1.4312
D	2.7428	2.5970	2.8968	0.5552	0.5125	0.6013

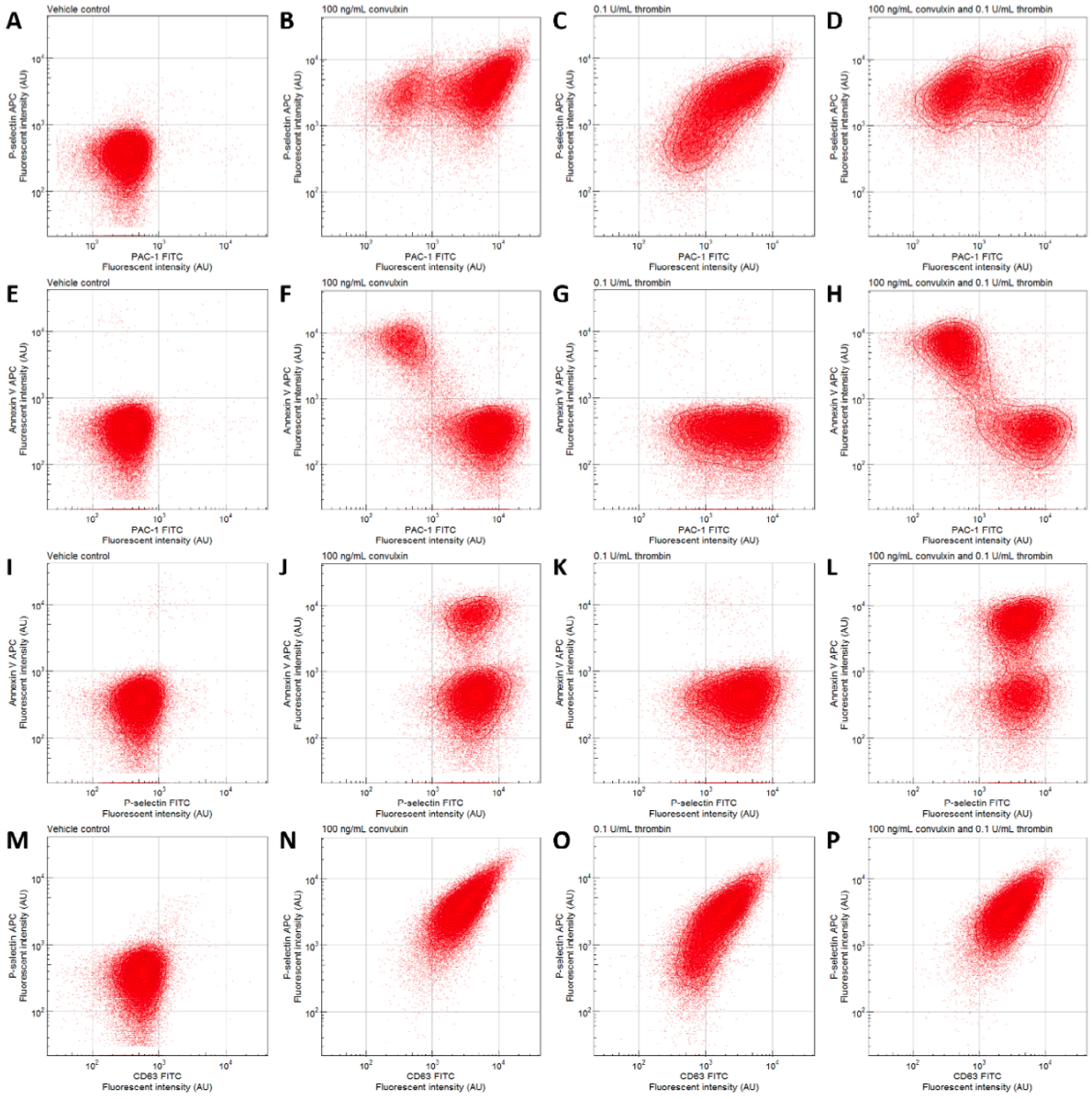

Figure C-11 Effect of stimulation of collective platelets with 100 ng/mL of convulxin and/or 0.1 U/mL of thrombin. Platelet activity measured with different panels of antibodies, blood from donor 1. Platelet stimulation in the presence of $CaCl_2$ (2 mM), GPRP (20 mM) and Rivaroxaban (0.1 μM in 0.01% DMSO). Platelets were selected with anti-CD42b (A-H) or anti-CD61 (I-P) and following the gating procedure described in section 2.3.5. Scatter plot of PAC-1 and P-selectin response (panel A) of platelets stimulated with **A)** vehicle control, **B)** only 100 ng/mL of convulxin, **C)** only 0.1 U/mL of thrombin and **D)** both 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Scatter plot of PAC-1 and Annexin V response (panel B) of platelets stimulated with **E)** vehicle control, **F)** only 100 ng/mL of convulxin, **G)** only 0.1 U/mL of thrombin and **H)** both 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Scatter plot of P-selectin and Annexin V response (panel C) of platelets stimulated with **I)** vehicle control, **J)** only 100 ng/mL of convulxin, **K)** only 0.1 U/mL of thrombin and **L)** both 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Scatter plot of CD63 and P-selectin response (panel D) of platelets stimulated with **M)** vehicle control, **N)** only 100 ng/mL of convulxin, **O)** only 0.1 U/mL of thrombin and **P)** both 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Data is shown as scatter plot with contour plot showing densities. This experiment was discussed in section 4.3.5. N=2 (also see Figure C-13). Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-10 and Table C-11.

Table C-10 Relative activation and confidence intervals of collective platelet activity stimulated with vehicle control compared to (dual) agonist stimulation with 100 ng/mL of convulxin and/or 0.1 U/mL of thrombin. Platelet activity measured with different panels of antibodies, blood from donor 1. This experiment was discussed in section 4.3.5. Antibody panels used were panel A: PAC-1 (FITC) and P-selectin (APC), panel B: PAC-1 (FITC) and Annexin V (APC), panel C: P-selectin (FITC) and Annexin V (APC)and panel D: CD63 (FITC) and P-selectin (APC).

Antibody panel A

Collective vehicle vs agonist stimulation response						
	PAC-1			P-selectin		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	8.3909	8.2893	8.4936	2.4438	2.4305	2.4571
0.1 U/mL thrombin	4.5347	4.4969	4.5728	1.6035	1.5978	1.6092
100 ng/mL convulxin and 0.1 U/mL thrombin	4.1325	4.0997	4.1657	3.9421	3.9116	3.9729

Antibody panel B

Collective vehicle vs agonist stimulation response						
	PAC-1			Annexin V		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	8.3765	8.2751	8.4792	2.4552	2.4419	2.4685
0.1 U/mL thrombin	4.5270	4.4892	4.5650	1.6110	1.6053	1.6166
100 ng/mL convulxin and 0.1 U/mL thrombin	4.1255	4.0927	4.1586	3.9606	3.9300	3.9914

Antibody panel C

Collective vehicle vs agonist stimulation response						
	P-selectin			Annexin V		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	8.0085	7.9104	8.1078	2.4495	2.4362	2.4629
0.1 U/mL thrombin	4.3281	4.2911	4.3654	1.6073	1.6016	1.6129
100 ng/mL convulxin and 0.1 U/mL thrombin	3.9442	3.9120	3.9768	3.9514	3.9208	3.9822

Antibody panel D

Collective vehicle vs agonist stimulation response						
	CD63			P-selectin		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	7.9064	7.8092	8.0048	2.4333	2.4199	2.4466
0.1 U/mL thrombin	4.2729	4.2361	4.3100	1.5966	1.5908	1.6024
100 ng/mL convulxin and 0.1 U/mL thrombin	3.8939	3.8618	3.9263	3.9252	3.8947	3.9559

Table C-11 Relative activation and confidence intervals of collective platelet activity stimulated with single agonist compared to dual agonist stimulation. Agonist concentration of 100 ng/mL of convulxin and/or 0.1 U/mL of thrombin. Platelet activity measured with different panels of antibodies, blood from donor 1. This experiment was discussed in section 4.3.5. Antibody panels used were panel A: PAC-1 (FITC) and P-selectin (APC), panel B: PAC-1 (FITC) and Annexin V (APC), panel C: P-selectin (FITC) and Annexin V (APC)and panel D: CD63 (FITC) and P-selectin (APC). Row names indicate single agonist stimulation (100 ng/mL convulxin depicts the RA of stimulation with 100 ng/mL of convulxin compared to 100 ng/mL of convulxin in addition of 0.1 U/mL of thrombin).

Antibody panel A

Collective single agonist stimulation vs dual agonist stimulation response						
	PAC-1			P-selectin		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	0.2833	0.2788	0.2879	7.4147	7.1403	7.6997
0.1 U/mL thrombin	0.5242	0.5172	0.5313	11.3003	10.8845	11.7319

Antibody panel B

Collective single agonist stimulation vs dual agonist stimulation response						
	PAC-1			Annexin V		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	0.2455	0.2418	0.2493	0.8133	0.8048	0.8219
0.1 U/mL thrombin	0.4543	0.4487	0.4600	1.2395	1.2276	1.2515

Antibody panel C

Collective single agonist stimulation vs dual agonist stimulation response						
	P-selectin			Annexin V		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	6.1729	5.7837	6.5884	1.0361	1.0234	1.0491
0.1 U/mL thrombin	11.4222	10.7083	12.1836	1.5791	1.5608	1.5977

Antibody panel D

Collective single agonist stimulation vs dual agonist stimulation response						
	CD63			P-selectin		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	2.6186	2.5087	2.7332	6.5610	6.3336	6.7965
0.1 U/mL thrombin	4.8453	4.6464	5.0527	9.9992	9.6550	10.3556

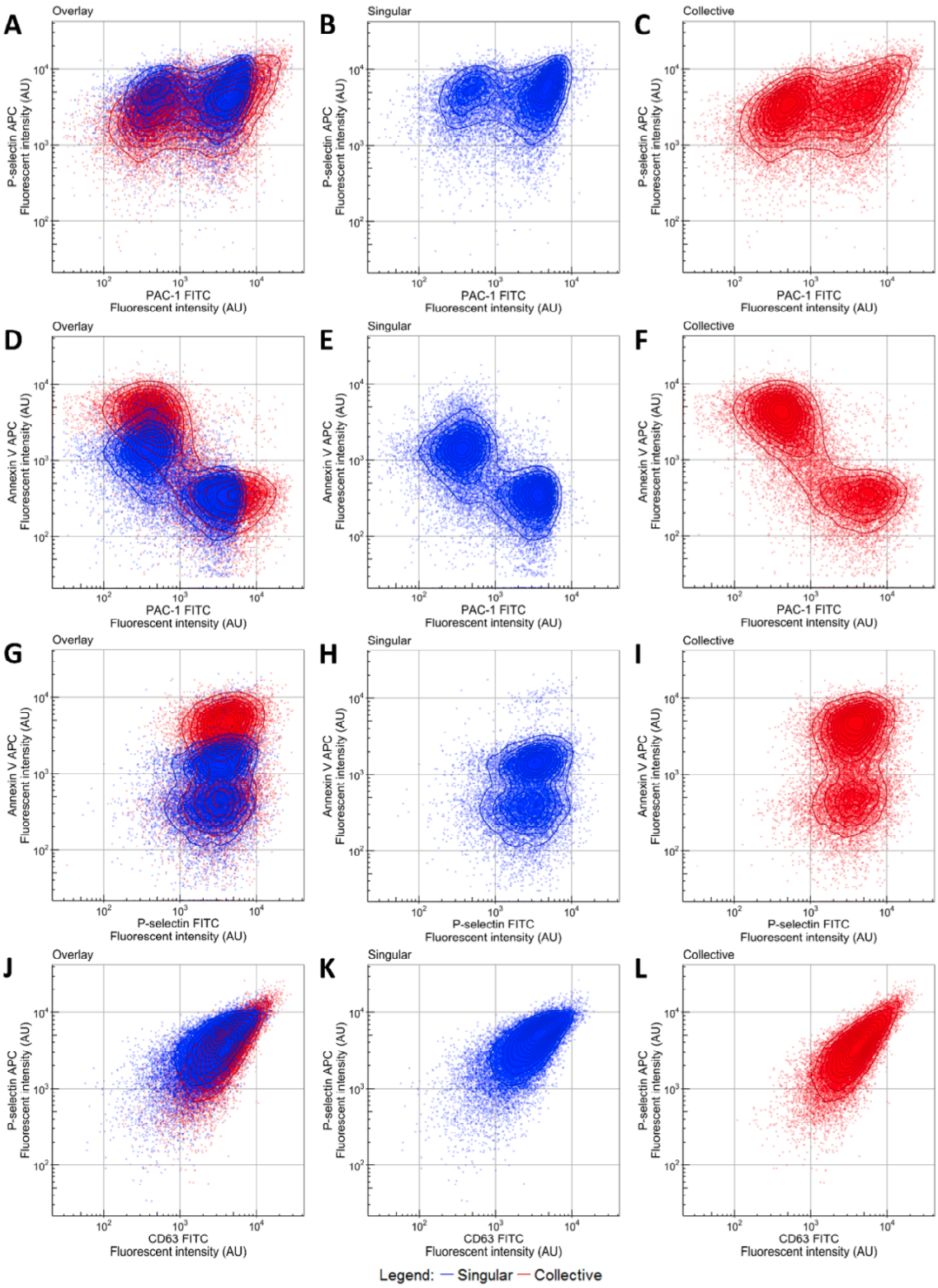

Figure C-12 Effect of stimulation of singular (blue) and collective (red) platelets with 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Platelet stimulation in the presence of $CaCl_2$ (2 mM), GPRP (20 mM) and Rivaroxaban (0.1 μM in 0.01% DMSO). Platelet activity measured with different panels of antibodies, blood from donor 8. Platelets were selected with anti-CD42b (A-F) or anti-CD61 (G-L) and following the gating procedure described in section 2.3.5. Scatter plot PAC-1 and P-selectin response (panel A) of **A)** overlay of singular and collective platelet response, **B)** only singular and **C)** only collective platelet response. Scatter plot PAC-1 and Annexin V response (panel B) of **D)** overlay of singular and collective platelet response, **E)** only singular and **F)** only collective platelet response. Scatter plot P-selectin and Annexin V response (panel C) of **G)** overlay of singular and collective platelet response, **H)** only singular and **I)** only collective platelet response. Scatter plot CD63 and P-selectin response (panel D) of **J)** overlay of singular and collective platelet response, **K)** only singular and **L)** only collective platelet response. Data is shown as scatter plot with contour plot showing densities. This experiment was discussed in section 4.3.5. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-12.

Table C-12 Relative activation and confidence intervals of singular platelet activity compared to collective in response to dual agonist stimulation with 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Platelet activity measured with different panels of antibodies, blood from donor 8. This experiment was discussed in section 4.3.5. Antibody panels used were panel A: PAC-1 (FITC) and P-selectin (APC), panel B: PAC-1 (FITC) and Annexin V (APC), panel C: P-selectin (FITC) and Annexin V (APC) and panel D: CD63 (FITC) and P-selectin (APC).

Singular vs collective response						
	Antibody 1 (FITC)			Antibody 2 (APC)		
Antibody panel	Relative activation	Confidence interval		Relative activation	Confidence interval	
A	0.6895	0.6717	0.7078	0.5189	0.4773	0.5642
B	0.9329	0.9180	0.9481	1.4971	1.4711	1.5236
C	2.6185	2.3964	2.8612	1.6925	1.6556	1.7302
D	2.6382	2.4276	2.8671	0.5955	0.5564	0.6374

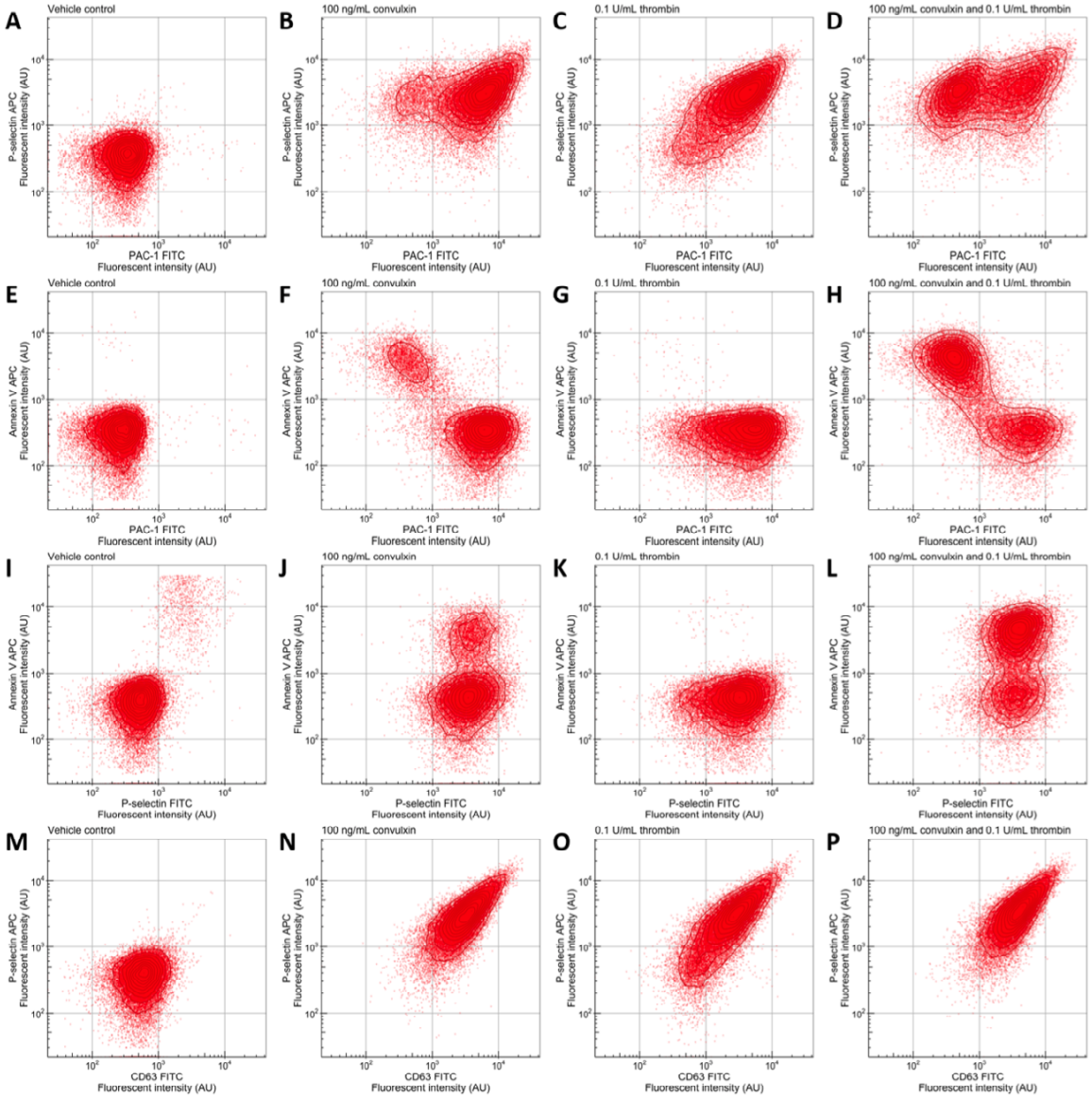

Figure C-13 Effect of stimulation of collective platelets with 100 ng/mL of convulxin and/or 0.1 U/mL of thrombin. Platelet activity measured with different panels of antibodies, blood from donor 8. Platelet stimulation in the presence of $CaCl_2$ (2 mM), GPRP (20 mM) and Rivaroxaban (0.1 μM in 0.01% DMSO). Platelets were selected with anti-CD42b (A-H) or anti-CD61 (I-P) and following the gating procedure described in section 2.3.5. Scatter plot of PAC-1 and P-selectin response (panel A) of platelets stimulated with **A)** vehicle control, **B)** only 100 ng/mL of convulxin, **C)** only 0.1 U/mL of thrombin and **D)** both 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Scatter plot of PAC-1 and Annexin V response (panel B) of platelets stimulated with **E)** vehicle control, **F)** only 100 ng/mL of convulxin, **G)** only 0.1 U/mL of thrombin and **H)** both 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Scatter plot of P-selectin and Annexin V response (panel C) of platelets stimulated with **I)** vehicle control, **J)** only 100 ng/mL of convulxin, **K)** only 0.1 U/mL of thrombin and **L)** both 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Scatter plot of CD63 and P-selectin response (panel D) of platelets stimulated with **M)** vehicle control, **N)** only 100 ng/mL of convulxin, **O)** only 0.1 U/mL of thrombin and **P)** both 100 ng/mL of convulxin and 0.1 U/mL of thrombin. Data is shown as scatter plot with contour plot showing densities. This experiment was discussed in section 4.3.5. N=2 Statistical analysis comprised of the relative activation (RA) with confidence intervals as seen in Table C-13 and Table C-14.

Table C-13 Relative activation and confidence intervals of collective platelet activity stimulated with vehicle control compared to (dual) agonist stimulation with 100 ng/mL of convulxin and/or 0.1 U/mL of thrombin. Platelet activity measured with different panels of antibodies, blood from donor 8. This experiment was discussed in section 4.3.5. Antibody panels used were panel A: PAC-1 (FITC) and P-selectin (APC), panel B: PAC-1 (FITC) and Annexin V (APC), panel C: P-selectin (FITC) and Annexin V (APC)and panel D: CD63 (FITC) and P-selectin (APC).

Antibody panel A

Collective vehicle vs agonist stimulation response						
	PAC-1			P-selectin		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	9.6558	9.4760	9.8391	2.2570	2.2407	2.2734
0.1 U/mL thrombin	6.3011	6.2097	6.3938	1.6351	1.6266	1.6436
100 ng/mL convulxin and 0.1 U/mL thrombin	3.6016	3.5643	3.6392	4.2570	4.2078	4.3067

Antibody panel B

Collective vehicle vs agonist stimulation response						
	PAC-1			Annexin V		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	9.7060	9.5255	9.8900	2.2688	2.2526	2.2852
0.1 U/mL thrombin	6.3339	6.2422	6.4269	1.6436	1.6353	1.6520
100 ng/mL convulxin and 0.1 U/mL thrombin	3.6203	3.5830	3.6580	4.2792	4.2300	4.3291

Antibody panel C

Collective vehicle vs agonist stimulation response						
	P-selectin			Annexin V		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	8.7425	8.5760	8.9123	2.1400	2.1234	2.1568
0.1 U/mL thrombin	5.7051	5.6192	5.7923	1.5504	1.5412	1.5596
100 ng/mL convulxin and 0.1 U/mL thrombin	3.2609	3.2247	3.2976	4.0364	3.9883	4.0850

Antibody panel D

Collective vehicle vs agonist stimulation response						
	CD63			P-selectin		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	8.6666	8.5011	8.8353	2.2517	2.2354	2.2681
0.1 U/mL thrombin	5.6556	5.5701	5.7423	1.6312	1.6228	1.6398
100 ng/mL convulxin and 0.1 U/mL thrombin	3.2326	3.1965	3.2691	4.2470	4.1979	4.2967

Table C-14 Relative activation and confidence intervals of collective platelet activity stimulated with single agonist compared to dual agonist stimulation. Agonist concentration of 100 ng/mL of convulxin and/or 0.1 U/mL of thrombin. Platelet activity measured with different panels of antibodies, blood from donor 8. This experiment was discussed in section 4.3.5. Antibody panels used were panel A: PAC-1 (FITC) and P-selectin (APC), panel B: PAC-1 (FITC) and Annexin V (APC), panel C: P-selectin (FITC) and Annexin V (APC)and panel D: CD63 (FITC) and P-selectin (APC). Row names indicate single agonist stimulation (100 ng/mL convulxin depicts the RA of stimulation with 100 ng/mL of convulxin compared to 100 ng/mL of convulxin in addition of 0.1 U/mL of thrombin).

Antibody panel A

Collective single agonist stimulation vs dual agonist stimulation response						
	PAC-1			P-selectin		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	0.2250	0.2198	0.2303	5.4467	5.2152	5.6885
0.1 U/mL thrombin	0.3447	0.3379	0.3517	7.5184	7.2010	7.8499

Antibody panel B

Collective single agonist stimulation vs dual agonist stimulation response						
	PAC-1			Annexin V		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	0.1794	0.1756	0.1834	1.0305	1.0138	1.0475
0.1 U/mL thrombin	0.2750	0.2700	0.2800	1.4225	1.4006	1.4448

Antibody panel C

Collective single agonist stimulation vs dual agonist stimulation response						
	P-selectin			Annexin V		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	3.2831	3.0489	3.5353	1.2721	1.2480	1.2966
0.1 U/mL thrombin	5.0310	4.6766	5.4124	1.7560	1.7239	1.7886

Antibody panel D

Collective single agonist stimulation vs dual agonist stimulation response						
	CD63			P-selectin		
Agonist	Relative activation	Confidence interval		Relative activation	Confidence interval	
100 ng/mL convulxin	3.4557	3.2044	3.7267	5.5525	5.3122	5.8035
0.1 U/mL thrombin	5.2955	4.9150	5.7055	7.6644	7.3350	8.0086

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