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Corresponding Author:	Chris Jones UNITED KINGDOM
Corresponding Author's Institution:	
Corresponding Author E-Mail:	c.i.jones@reading.ac.uk
Order of Authors:	Joane Dunster
	Jo Mitchell
	Yasmin Mohammed
	Kirk Taylor
	Jon Gibbins
	Chris Jones
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TITLE:

Kinetx: A Combined Flow Cytometry Assay and Analysis Software Framework to Quantitatively Measure and Categorize Platelet Activation in Real-time.

AUTHORS AND AFFILIATIONS:

Joane L. Dunster[1*], Jo L. Mitchell[1,2*], Yasim M. M. Mohammed[1], Kirk A. Taylor[1], Jon M. Gibbins[1], Christopher I Jones[1#]

[1]ICMR, School of Biological Sciences, University of Reading, Reading, Berkshire, England, United Kingdom

[2]Institute of Cardiovascular Sciences, University of Birmingham, Edgbaston, Birmingham, England, United Kingdom

* These authors contributed equally.

Email addresses of co-authors:

Joane L. Dunster	(j.l.dunster@reading.ac.uk)
Jo L. Mitchell	(j.l.mitchell@reading.ac.uk)
Yasim M. M. Mohammed	(y.m.m.mohammed@pgr.reading.ac.uk)
Kirk A. Taylor	(k.a.taylor@reading.ac.uk)
Jon M. Gibbins	(j.m.gibbins@reading.ac.uk)
Christopher I. Jones	(c.i.jones@reading.ac.uk)

[#]Corresponding author:

Christopher I. Jones	(c.i.jones@reading.ac.uk)

SUMMARY:

Platelets react rapidly to a range of stimuli. This paper describes a real-time flow cytometry-based platelet function assay and a newly developed bespoke open-source software (Kinetx) to enable quantitative kinetic measurements of platelet granule release, fibrinogen binding, and intracellular calcium flux.

ABSTRACT:

Platelets react rapidly to vascular injury and undergo activation in response to a range of stimuli to limit blood loss. Many platelet function tests measure endpoint responses after a defined time period and not the rate of platelet activation. However, the rate at which platelets convert extracellular stimuli into a functional response is an essential factor in determining how efficiently they can respond to injury, bind to a forming thrombus, and signal to recruit other platelets. This paper describes a flow cytometry-based platelet function assay that enables simultaneous data acquisition and sample stimulation and utilizes newly developed bespoke open-source software (Kinetx) to enable quantitative kinetic measurements of platelet granule release, fibrinogen binding, and intracellular calcium flux. Kinetix was developed in R so that users can alter parameters such as degree of smoothing, identification of outlying data points, or time scales. To aid users unfamiliar with the R environment, Kinetix analysis of data can be performed

by a single command. Together, this allows real-time platelet activation metrics, such as rate, acceleration, time to peak-rate, time to peak-calcium, and qualitative shape changes, to be accurately and reproducibly measured and categorized. Kinetic measurements of platelet activation give a unique insight into platelets' behavior during the first stages of activation and may provide a method of predicting the recruitment of platelets into a forming thrombus.

INTRODUCTION:

Platelets play a central role in hemostasis and generate a rapid and multifaceted response to vascular injury[1,2]. Current platelet function tests measure various aspects of platelet reactivity, representative of their hemostatic actions *in vivo*. Traditionally, platelet function has been assessed using endpoint assays that measure fibrinogen binding, granule release, or platelet aggregation in platelets stimulated for long enough to achieve maximal activation[3,4]. These tests do not take into account the time taken for platelets to convert extracellular stimuli to intracellular signals and calcium flux and subsequently degranulate and bind to the growing thrombus. Blood flows at a shear rate of up to 20 dynes/cm^3 in veins and 80 dynes/cm^3 in large arteries[5], emphasizing the need for platelets to counteract this rapid rate of flow by detecting, processing, and responding to extracellular stimuli rapidly to bind into a forming thrombus and support its ongoing growth.

The rate of platelet activation can vary independently of the maximum extent of platelet activation. Integrin-linked kinase (ILK) is a protein involved in regulating β1 and β3 integrins in platelets[6,7]. Inhibition or specific deletion of ILK *in vivo* demonstrated that the rate, but not the maximal extent of platelet activation, was affected in the absence of functional ILK[8]. Differences between the rate of aggregation and maximum level of aggregation were also identified in mice deficient in CALDAG GEFI[9,10], a signaling molecule involved in the regulation of inside-out signaling via RAP1[11].

These findings demonstrate that platelet rate and maximal activation levels can be autonomous and that measurements of maximal activation may not be descriptive of platelet behavior up to this point. These differences in the time taken for platelets to become activated may profoundly affect their initial binding to the growing thrombus, impacting the architecture and overall size of the thrombus. These variations between platelet rate and maximal activation highlight the need for an assay to accurately measure the rate of platelet activation and can be used to detect variances within the population.

This paper describes a method that accurately measures platelet activation and calcium flux in real-time and calculates a range of metrics, including the rate at which platelets become activated and the time taken for platelets to reach the maximum rate of activation and maximum calcium flux. These kinetic measurements of platelet activation give a better insight into the behavior of platelets during the first stages of thrombus formation and provide a method for predicting how quickly platelets can be recruited into a forming thrombus.

PROTOCOL:

This protocol used human whole blood and platelets from healthy donors with no underlying health conditions and not taking any medication known to interfere with platelet function after written informed consent and was approved by the University of Reading Research Ethics Committee.

1. Collection of blood and preparation of platelet-rich plasma

1.1. Collect the peripheral blood in a vacutainer containing 3.2% sodium citrate

1.2. Discard the first 3 mL.

1.3. Let the blood rest at 30 °C for 30 min prior to centrifugation.

1.4. Prepare the platelet-rich plasma (PRP) by centrifugation at 140 *x g* for 20 min at room temperature before incubation at 37 °C for the assay duration, which was finished within 60 min of centrifugation.

2. Instrument preparation

NOTE: A non-pressurized fluidics system is essential for real-time flow cytometry as platelets are stimulated simultaneously as the flow cytometer records events.

2.1. Use a non-pressurized flow cytometer to carry out real-time flow cytometry.

2.2. To perform the assay at 37 °C, attach a mouse dissection mat to the stage to place the 96-well plate. Heat the mat to 37 °C for the duration of the assay.

2.3. Place a 96-well plate on top of the heated mat to allow for continuous incubation at 37 °C (**Figure 1**)

3. Assay preparation

3.1. Fibrinogen binding and P-selectin expression

3.1.1. Coat a polypropylene 96-well round-bottomed, non-treated microtitre plate with 5% bovine serum albumin (BSA) in Hepes buffered saline (HBS; 10 mM Hepes pH 7.4; 150 mM NaCl, 5 mM KCl, 1 mM $MgSO_4 \cdot 7H_20$) overnight at 4 °C .

3.1.2. Prepare the antibody mix in HBS containing 5 mM glucose (HBS-G). Add fluorescein isothiocyanate (FITC)-labelled anti-fibrinogen antibody, (FITC-FGN; dilution 1: 37.5 (v/v)) and allophycocyanin (APC)-labelled anti-P-selectin (APC-CD62P; dilution 1: 37.5 (v/v)) to HBS-G (final volume: 225 µL) and incubate in a 96-well plate at 37 °C .

3.1.3. Immediately before collecting events, add PRP to the HBS-G-antibody mix at a dilution of 1:600 (v/v).

NOTE: A low volume of PRP is required to ensure the event limit is not exceeded and reduce platelet aggregation in the sample. Platelet dilution was examined in pilot studies and does not affect activation rate.

3.1.4. Incubate the activation mix containing FITC-FGN, (dilution 1: 37.5 (v/v)) and APC-CD62P (dilution 1: 37.5 (v/v)) and agonist at 37 °C. The agonists include thrombin (thr; 0.0012–1 U/mL) in the presence of Gly-Pro-Arg-Pro peptide (GPRP; 1.18 mM), cross-linked collagen related peptide (CRP-XL; 0.004–10 µg/mL), adenosine diphosphate (ADP; 0.04–4 µM), epinephrine (epi; 20–200 µM), 9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin F2α (U46619; 0.02–5 µM), and thrombin receptor activator peptide 6 (TRAP-6; 0.04–10 µM).

3.2. Calcium flux

NOTE: This assay was performed using a commercial calcium assay kit (**Table of Materials)**

3.2.1. Coat a polypropylene 96-well round-bottomed, non-treated microtitre plate with 5% BSA in Hepes buffered saline overnight at 4 °C.

3.2.2. To prepare the calcium assay dye, add 100 mL of calcium assay buffer and 2 mL probenecid stock solution (250 mM) to one bottle of calcium reagent to obtain 2x calcium reagent loading solution.

3.2.3. To prepare Fluo-4/HBS-G, dilute the calcium assay dye with HBS-G at a 1:1 ratio, filter through a 0.22 µM filter. Add 225 µL of Fluo-4/HBS-G to the appropriate wells of a 96-well plate at 37 °C.

3.2.4. Incubate PRP at 37 °C for 30 min at a 1:1 dilution with Fluo-4/HBS-G.

3.2.5. Prepare the activation mix in Fluo-4/HBS-G and incubate at 37 °C. The agonists include thrombin (1 U/mL) in the presence of GPRP, CRP-XL (0.5 µg/mL) ADP (1 µM), epinephrine (20 µM), U46619 (1 µM), and TRAP-6 (1 µM).

3.2.6. Immediately before collecting events, add PRP/Fluo-4/HBS-G to the 225 µL of Fluo-4/HBS-G mix at a final PRP dilution (after agonist addition) of 1: 600 (v/v; 0.5 mL in 300 mL of the final volume).

4. Assay procedure and data collection

4.1. Set the flow cytometer to a flow rate on 'slow' with an average number of 1000 events per second (eps) and a maximum of 3000 eps. Set the threshold to 20000 to avoid recording cell debris.

4.2. Gate the platelet population using forward scatter (FSC), and side scatter (SSC) plots. For data collection, plot FSC-A vs. SSC-A to accurately gate the platelet population and FL1 or FL4 vs. time to record the fluorescent events over time.

4.3. The flow cytometer takes 14 s to begin recording events; after this, let the instrument record events in each well for another 5 s before which 75 µL of the activation mix was added rapidly using a gel loading tip to ensure spontaneous mixing (**Figure 1**).

4.4. Collect the events for 5 min and set up the plots to record the 488 (FL1; 533/30 filter – FITC-fibrinogen/FLUO-4) channel vs. time and the 633 (FL4; 675/25 filter – APC-P-selectin) channel vs. time.

4.5. Save the plot data for the 488 and 633 channels from each well as CSV files.

5. Data analysis

5.1. Download the latest version of Kinetx[12].

NOTE: The download includes all code required to analyze the data generated from the assay and is accompanied by a test set of experimental data from ten donors and outputs generated from their analysis (these results can be regenerated and validated via the following procedures) (**Figure 2**).

5.2. Set up R environment

5.2.1. Use the programming language R via the RStudio environment. Install R[13], RStudio[14] and the following packages: ggplot2, dplyr, plotrix, minpack.lm, tidyverse, psych. Ensure that all pre-requisite packages have been successfully installed.

5.2.2. Create Metadata: Place a text file (called wellData.txt) inside the same directory as the data. It describes the agonists, their concentrations, any antibodies, and the assay (function or calcium) used to generate each CSV file.

5.2.3. Place a second text file ('IsotypeData.txt') in the same directory and defines the isotypes. The files provided in the download ('data/wellData.txt' and 'data/IsotypeData.txt') can act as a template, being easily adaptable to alternative agonists, inhibitors, and concentrations.

5.2.4. Analyze the data: Kinetx comprises three functions as defined in steps 5.2.5–5.2.7.

5.2.5. kinetxProcess.R: This function processes the raw functional flow cytometry data. To run type kinetxProcess('data location','where to put outputs','FL1' (for FITC-anti-fibrinogen, or 'FL4' (for APC-CD62P)

5.2.6. kinetxProcessCalcium.R: This function analyzes and labels the calcium flux data. To run type kinetxProcessCalcium('data location','where to put outputs','FL1')

5.2.7. kinetxSummary.R: This function summarizes the data. To run type kinetxSummary('data location','where to put outputs','FL1').

5.2.8. To run all of the above functions (5.2.5–5.2.7) on the test data, use the script (test.R).

NOTE: The first two functions produce figures for each donor that shows the raw data plus the fitted curves. All outputs and a spreadsheet that summarises the data are placed in the output location.

REPRESENTATIVE RESULTS:

The analysis of the functional real-time flow cytometry reveals differences in the shape of activation curves between agonists and between donors

Data analysis of the functional data (P-selectin exposure and fibrinogen binding) using Kinetx removes data points that are out of reasonable ranges (>200000 or <1) and <2.80 s (zero time being set to this point), normalizes data to the median of the isotypes and fits a smooth moving average curve (via function loess). Kinetx also assigns metrics and categories are assigned based on the fitted smooth lines shape, absolute values, and rates of change as follows: (1) RoC = average rise in smoothed line over the first 120 s; (2) label = categorizes responses as fast, medium, or slow being set according to the cut-offs of 40 and 80 for FL1 and 5 and 10 for FL4; (3) EarlyRoC = average rise in smoothed line over the first 20 s; (4) shape = low responders, increasing, linear or decreasing (**Figure 3A**) - Low responders being determined as FL1 < 4000 or FL4 < 1000, the labels increasing, linear and decreasing are determined based on: shapeMetric = RoC(120 seconds)/EarlyRoC, where increasing shapeMetric >2, linear shapeMetric between 0.9–2 and decreasing shapeMetric <0.9; (5) Produces a spreadsheet summarising the data by providing the level and rate of change at 0 s, 10 s, 20 s, 30 s, 40 s, 60 s, 90 s, 120 s, 180 s, isotype median, the maximum and minimum rates of change and acceleration and all labels and categories.

Platelet fibrinogen binding and P-selectin exposure over 5 min following activation in response to a single concentration of the agonists ADP, TRAP-6, CRP-XL, epinephrine, and U46617, was analyzed in 30 donors. The metrics for fibrinogen binding and P-selectin exposure in response to different agonists show considerable variation in the shape of the response (**Figure 3**). Platelets stimulated with TRAP-6 and ADP showed a more rapid acceleration in fibrinogen binding, which for many donors slowed at later time points (**Figure 3B**). Fibrinogen binding in response to CRP-XL, thrombin, and epinephrine was more likely to show an increasing or linear rate of response. Many donors showed a relatively long lag time for CRP-XL before the rate of fibrinogen binding accelerated rapidly. Response to U46619 was often categorized as low. In contrast, P-selectin

exposure was more likely to show a linear or increasing rate of response to all agonists except U46619 which again showed low response levels (**Figure 3C**).

Intracellular calcium flux during platelet activation can be accurately measured using real-time flow cytometry

Data analysis of the calcium flux assay removes data points that are out of reasonable ranges (< 2.80 s zero time being set to this point) and then fits a smooth moving average curve (via function loess). Kinetx assigns metrics, and categories are assigned based on the fitted smooth lines shape, absolute values, and rates of change as follows: (1) category - that describes the shape of the calcium response based on the early rise, the maximum, and the final drop from maximum, described as linear, peak or sustained (**Figure 4A**); (2) rate - that describes the rate of change in the fast 30 s and is defined as no change, slow, medium or fast (with cut off at 10, 40 and 80). Kinetx also produces a spreadsheet summarizing the data by providing the level and rate of change at 0 s, 10 s, 20 s, 30 s, 40 s, 60 s, 90 s, 120 s, 180 s, the maximum and minimum rates of change and acceleration and all labels and categories, as well as producing a figure of the data for each donor. Examples of calcium flux measured by this method in 30 individuals again showed different patterns of activation between individuals and agonists (**Figure 4B**). CRP-XL gave predominantly sustained calcium response, whereas the response to TRAP-6 most often showed a peaked response. All other agonists generated a predominantly linear response.

Real-time flow cytometry identifies differences in maximum levels of activation in response to different agonists and concentrations

Results demonstrate that the real-time flow cytometry assay has the capacity to detect differences in the maximum levels of fibrinogen binding (**Figure 5A**) and P-selectin exposure (**Figure 5D**), in response to stimulation via different receptors (10 µg/mL CRP-XL vs. 3 U/mL thrombin $P < 0.001$; 10 µg/mL CRP-XL vs. 1 U/mL thrombin $P < 0.001$). They also show that the assay is sensitive to differences in agonist concentration, where levels of maximum fibrinogen binding and P-selectin exposure are increased in response to greater agonist concentrations (fibrinogen binding - CRP-XL - 0.004 µg/mL vs. 0.4, 5, 10 µg/mL, $P < 0.01$; thrombin - 0.0012 U/mL vs. 1 and 3 U/mL, $P < 0.01$; ADP - 0.04 µg/mL vs. 1 and 4 µM, $P < 0.05$).

The real-time flow cytometry assay is sensitive enough to detect variability amongst donors in platelet activation rate

The maximum rate of platelet activation was determined by fitting a smoothed moving average (loess) to the fluorescence data and then using this to calculate the maximum rate of fibrinogen binding and P-selectin exposure (**Figure 5B**,**E**). The data demonstrate a considerable variation between individual donors, and the flow cytometry assay is sensitive enough to detect this variation. Results indicate that increasing agonist concentration has little effect on the maximum rate of fibrinogen binding (**Figure 5B**). In contrast, the maximum rate of P-selectin exposure increases with greater thrombin and CRP-XL concentration, but not with increasing concentrations of ADP (**Figure 5E**).

Variations in the lag time of platelet activation can be detected using real-time flow cytometry

The time to the maximum rate of platelet activation was determined by calculating the time

points at which the maximum rate of fibrinogen binding and P-selectin exposure were reached (**Figure 5C**,**F**). The maximum rate of fibrinogen binding and P selectin exposure varies between donors, particularly at lower agonist concentrations. Stimulation of platelets with increasing concentrations of CRP-XL shows higher agonist concentrations take less time to reach the maximal rate of fibrinogen binding with less variability between donors; however, this trend is not seen with either thrombin or ADP stimulation (**Figure 5C**). The assay demonstrates that in some circumstances, increasing the agonist concentration decreases the time taken for platelet activation to commence (lag time) without altering the maximum rate of platelet activation and that both of these measures can be assessed with this assay.

FIGURE AND TABLE LEGENDS:

Figure 1: Wet-lab real-time flow cytometry. (**A**) anticoagulated whole blood was (**B**) centrifuged to obtain platelet-rich plasma (PRP). (**C**) Diluted PRP containing appropriate antibodies or calcium indicator dyes was placed in a 96-well plate, loaded onto a non-pressurized flow cytometer, and maintained at 37 °C using a dissection mat. Samples were stimulated during acquisition with an activation mix containing antibodies or calcium dye and agonist, rapidly added to the well using a gel loading tip to ensure spontaneous mixing. (**D**) Events were collected for 5 min, and CSV files of the data for each well were saved.

Figure 2: Schematic of the data analysis. (**A**) R environment was configured, and metadata was created. (**B**) Outlying data were removed, and a smooth moving average (loess) curve was fitted. (**C**) Response type and (**D**) response shape were defined. (**E**) Metrics were calculated, exported and figures generated for each donor.

Figure 3: Representative output of Kinetx analysis of real-time platelet fibrinogen binding and P-selectin expression. (**A**) As well as providing metrics such as maximum rate of change (RoC) and the RoC at various time points, Kinetx also categorizes the shape of the curve to provide additional information on the activation of platelets that is hard to capture in a single metric. Representative data for 30 individuals show the typical variation seen in real-time platelet (**B**) fibrinogen binding and (**C**) P-selectin expression in response to the agonists ADP, TRAP-6, CRL-XL, epinephrine, and U46617.

Figure 4: Representative output of Kinetx analysis of real-time platelet Calcium Flux. (**A**) As well as providing metrics such as maximum rate of change (RoC) and the RoC at various time points, Kinetx also categorizes the shape of the calcium flux curve to provide additional information on the activation of platelets that is hard to capture in a single metric. Representative data for 30 individuals show the typical variation seen in real-time platelet (**B**) Calcium Flux in response to the agonists ADP, TRAP-6, CRL-XL, epinephrine, and U46617.

Figure 5: Agonist concentration determines maximum platelet activation but not the rate of platelet activation. Maximum platelet activation – fibrinogen binding (**A**) or P-selectin exposure (**D**) – was significantly positively altered by agonist (CRP-XL, thrombin, or ADP) concentration. The rate of platelet activation (**B** and **E**) and the time to the maximal rate of platelet activation (**C** and **F**) showed either no relationship or a weaker relationship with changing agonist

concentration. P-values show the significance over the whole concentration range (Kruskal-Wallis test), and asterisks indicate a significant difference (multiple comparison test) of individual values from the lowest concentration. * $P < 0.05$, ** $P < 0.01$.

DISCUSSION:

The rate at which platelets detect, process, and respond to activating stimuli may be an essential determinant for thrombus formation. Previous studies have found that inhibition of signaling elements that impact the rate, but not the final extent of platelet activation, results in the formation of unstable thrombi[8]. Many platelet function assays measure the extent of platelet activation and aggregation in response to different conditions and treatments; however, these do not consider the rate at which platelets become activated and the time taken for this complex process to occur[3,4]. The innovative flow cytometry-based assays developed here reproducibly monitor platelet activation over time and translate this into a range of metrics to calculate the maximum rate of platelet activation and the time taken for platelets to reach this maximum rate and become fully activated.

The data presented highlights the real-time assay's capacity to identify variations between different responses to agonist type and concentration and between individuals. Many previous reports have shown that platelet reactivity is highly variable between normal individuals[15,16], indicating that the rate of platelet activation may also vary significantly within the population. Data from this study demonstrates that the rate of degranulation and fibrinogen binding to platelets appears to be even more variable than the maximum levels of platelet activation. This shows that real-time flow cytometry can also be utilized as a valuable and reliable tool to identify variances in platelet rate in the population and detect the effects of different inhibitory or pro-activatory agents on platelet rate as an additional means of measuring platelet function.

When platelet function is measured using endpoint assays, comparisons can be made on the extent of fibrinogen binding or granule release between different agonists. The loess curves comparing fibrinogen binding over the first 5 min of platelet activation demonstrate the ability of the real-time assay to tease out more detail in the differences in platelet activation kinetics that single endpoint assays cannot measure.

The data collected from the real-time assay demonstrates that the speed at which fibrinogen binds to platelets follows a slightly different pattern, depending on the activating pathway (**Figure 3**). In order to accurately assess platelet activation kinetics, it is essential to use a non-pressurized flow cytometer that enables simultaneous data acquisition and sample stimulation. It is also essential that the agonists are added rapidly to ensure near-instantaneous and complete mixing of agonist and platelets. Stimulation with thrombin ADP and epinephrine produces a similar curve representing a rapid initiation of signaling in response to receptor sensitization resulting in fibrinogen binding to platelets at a quick and steady rate. In contrast, platelets stimulated by CRP-XL are initially very slow to bind fibrinogen following initial receptor sensitization; however, the rate of fibrinogen binding is then rapidly increased after this initial delay. Platelet stimulation with U46619, a mimetic of TXA2, results in a quick initial rate of fibrinogen binding, which

decreases rapidly to a very slow rate leading to only a slight and steady increase in fibrinogen binding over time.

Kinetx was designed to be open-source, reproducible, and easy to implement in order to get around problems with proprietary software, such as cost and inflexibility. As such, it needed to be developed with software that was non-propriety. R was chosen as it is widely used by biologists, easy to install, cost-free, and open-source. This open-source environment allows users proficient in R to alter parameters such as degree of smoothing, identification of outlying data points, or time scales. However, to aid researchers who are unfamiliar with R Kinetix was also developed so that analysis can be performed via a single command (either kinetxProcess or kinetxProcessCalcium, depending on the data being analyzed). The Kinetix software demonstrated here can calculate a range of metrics, including values for the maximum levels, rate, and acceleration of fibrinogen binding or P-selectin exposure and the time points at which these maximums occur. The kinetics of platelet activation in response to different stimulatory pathways can be more accurately compared using these numbers.

Comparisons between different agonist stimulations in the maximum levels of fibrinogen binding and the rates of fibrinogen binding to platelets are good examples of where the rate of platelet activation can vary independently of maximum binding. Comparing maximum fibrinogen binding after 5 min of stimulation with 0.04 μM ADP (3.85 LogFU) and 0.04 μg/mL CRP-XL (3.70 LogFU), both agonists have resulted in a similar amount of bound fibrinogen (**Figure 5A**). The maximum rates of fibrinogen binding show an even more minor difference (0.04 μM ADP - 0.0050 LogFU/minute; 0.04 μg/mL CRP-XL - 0.0054 LogFU/min), indicating that the overall rate of fibrinogen binding is similar between these two stimulations (**Figure 5B**). However, when the lag time of platelet activation is compared, there is a clear difference showing CRP-XL (117 s to maximum rate) accelerating at a much slower rate than ADP (47 s to maximum rate) (Figure 5C). Thus, it becomes clear that ADP stimulation results in a much faster initial response ($P < 0.001$) when compared to CRP-XL. When observed together, these measurements of platelet kinetics in response to two different agonists describe a rapid initial response to ADP stimulation which accelerates slowly and remains at a steady rate. In contrast, CRP-XL stimulation results in a slow initial rate of activation, which accelerates rapidly at a much later time, eventually leading to a similar overall rate and levels of fibrinogen binding as ADP. These differences between the maximum levels, rate, and acceleration of fibrinogen binding demonstrate that a number of parameters are involved in measuring how quickly platelets become activated. The real-time assay and Kinetx analysis can measure and compare these parameters, describe the time taken from receptor sensitization to the platelet response, and compare this between different signaling pathways.

Increasing agonist concentration does not have a significant effect on the rate of fibrinogen binding to platelets. However, the time taken to reach the maximum rate of fibrinogen binding decreases as agonist concentration increases, suggesting that platelets bind fibrinogen at a steady rate and receptor saturation plays a more prominent role in how quickly platelets bind fibrinogen.

The calcium flux assay and analysis described is a quick and easy to perform calcium assay that can be incorporated as extra samples in the real-time assay allowing for the analysis of calcium and platelet fibrinogen binding and P-selectin in the same run of samples. The bespoke analysis package provides an in-depth assessment of calcium flux kinetics, including the shape of the response, maximum response, and time to maximum response. These parameters can then be compared for variation between donors and in response to various pharmaceutical agents. Calcium flux in platelet has been previously studied in platelets using a variety of flow cytometry-based assays[17–19]. Aliotta et al.[14], describe an elegant assay capable of analyzing the kinetics of multiple intracellular ions. The calcium assay presented here builds upon these previously published assays by including the analysis package allowing greater flexibility, a more in-depth exploration of the data with the benefit of high-throughput analysis for multiple donors in a short timeframe.

Previous studies have demonstrated that the inhibition or absence of certain signaling molecules results in an altered platelet activation rate, which directly translates to thrombus formation[8,9]. In the past, platelet activation kinetics could be measured by flow cytometry over a number of fixed time points which can then be used to calculate and compare, for example, the rate of GPIIbIIIa externalization in response to different agonists[20]. The real-time assay and Kinetx analysis described in this paper provide a simple, freely available, and accurate method for measuring both rate and endpoint of platelet activation from resting platelets. This is likely important in identifying physiologically relevant variations in platelet function that may be missed when only endpoint readings are measured.

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DISCLOSURES:

The authors have nothing to disclose.

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13. R at <https://www.r-project.org/> (2021).
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Figure 1

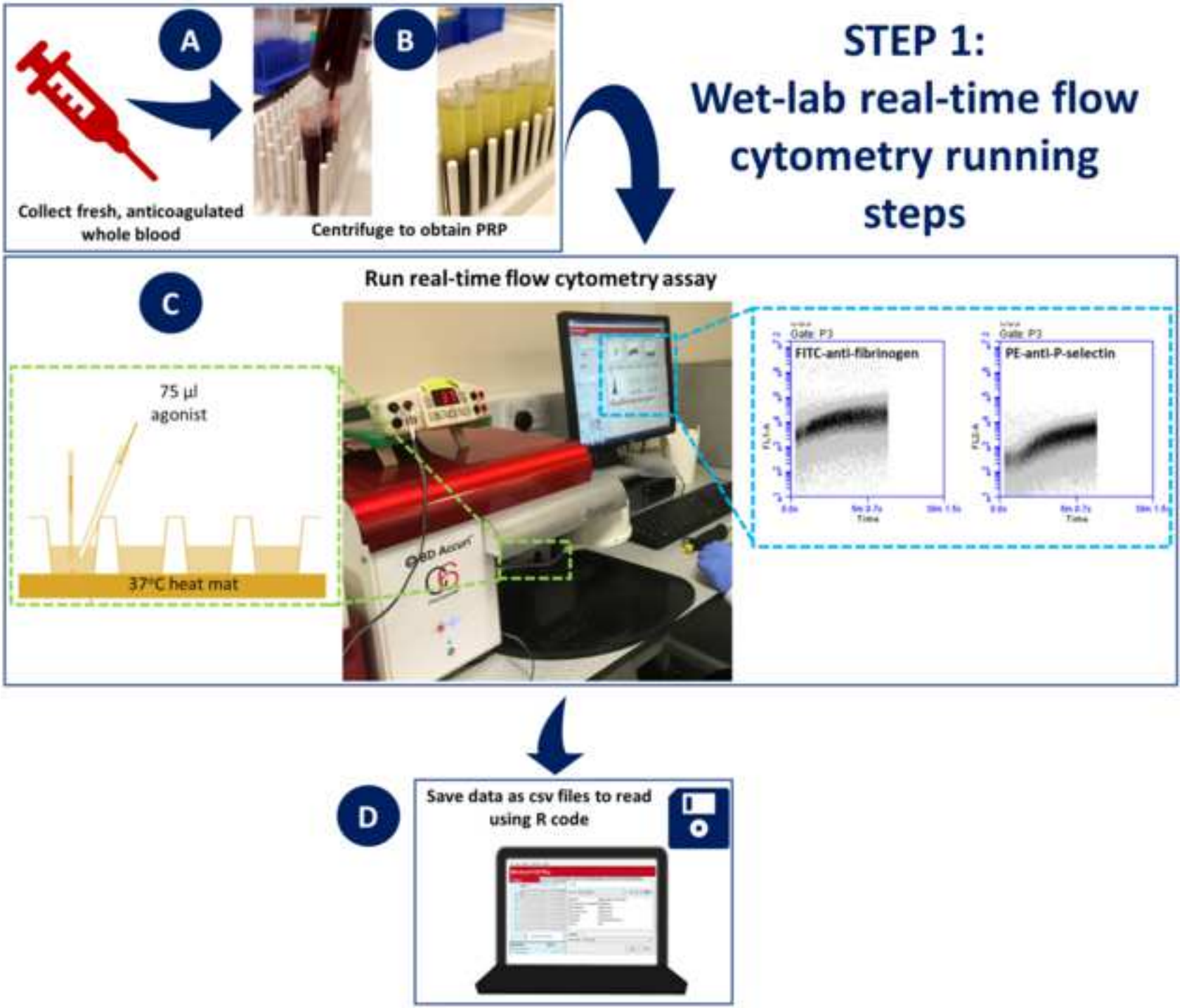


Figure 2

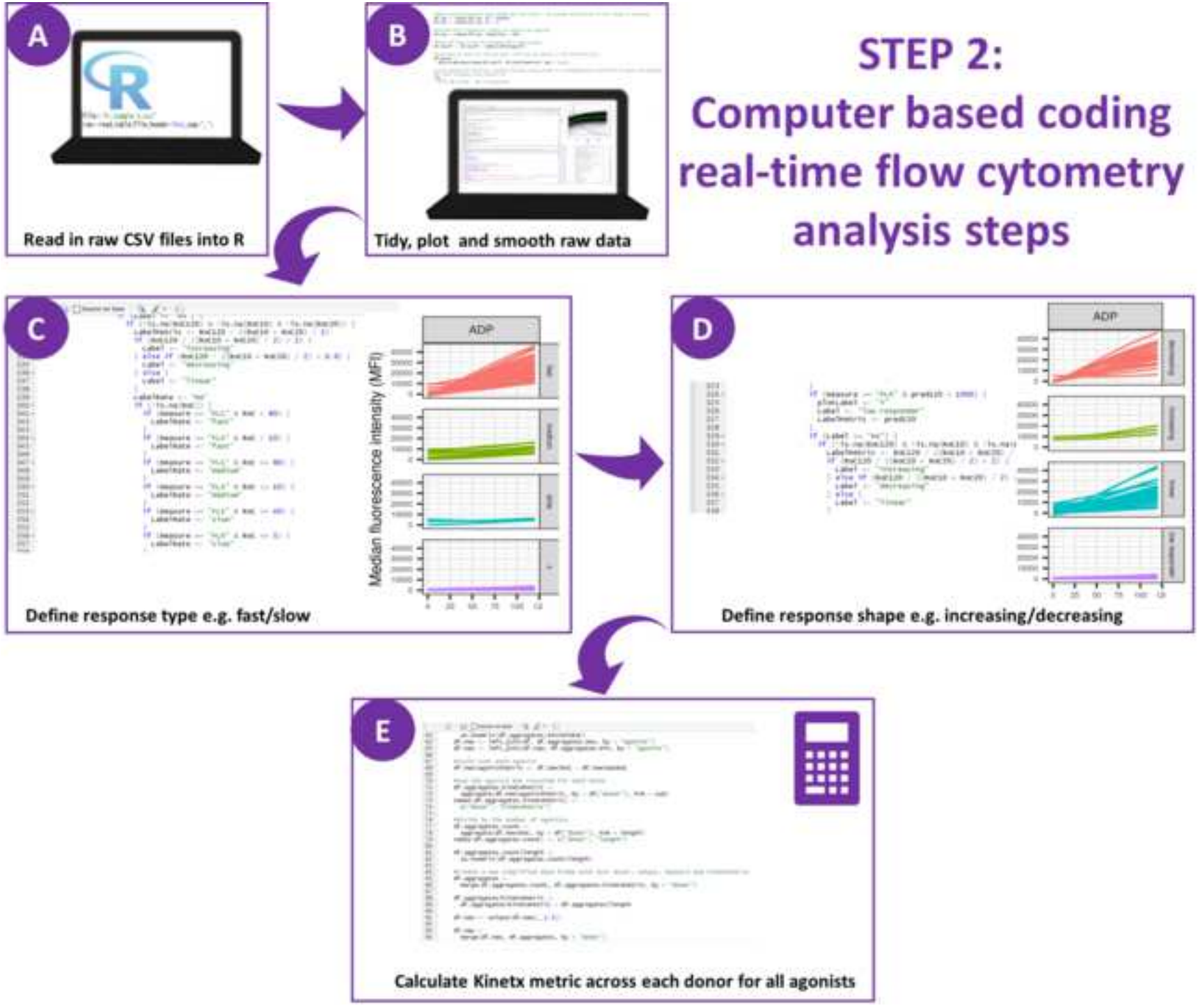


Figure 3

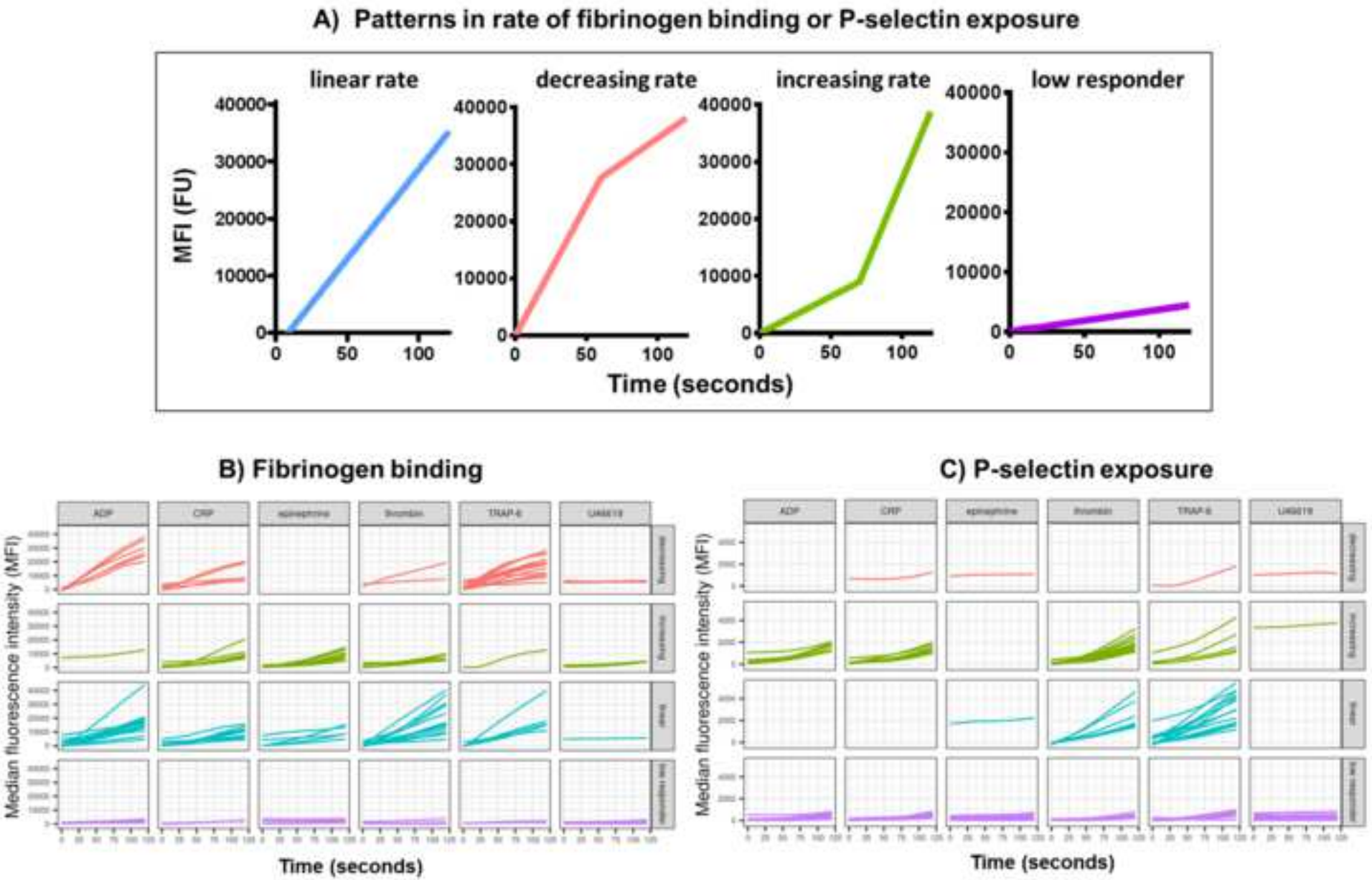


Figure 4

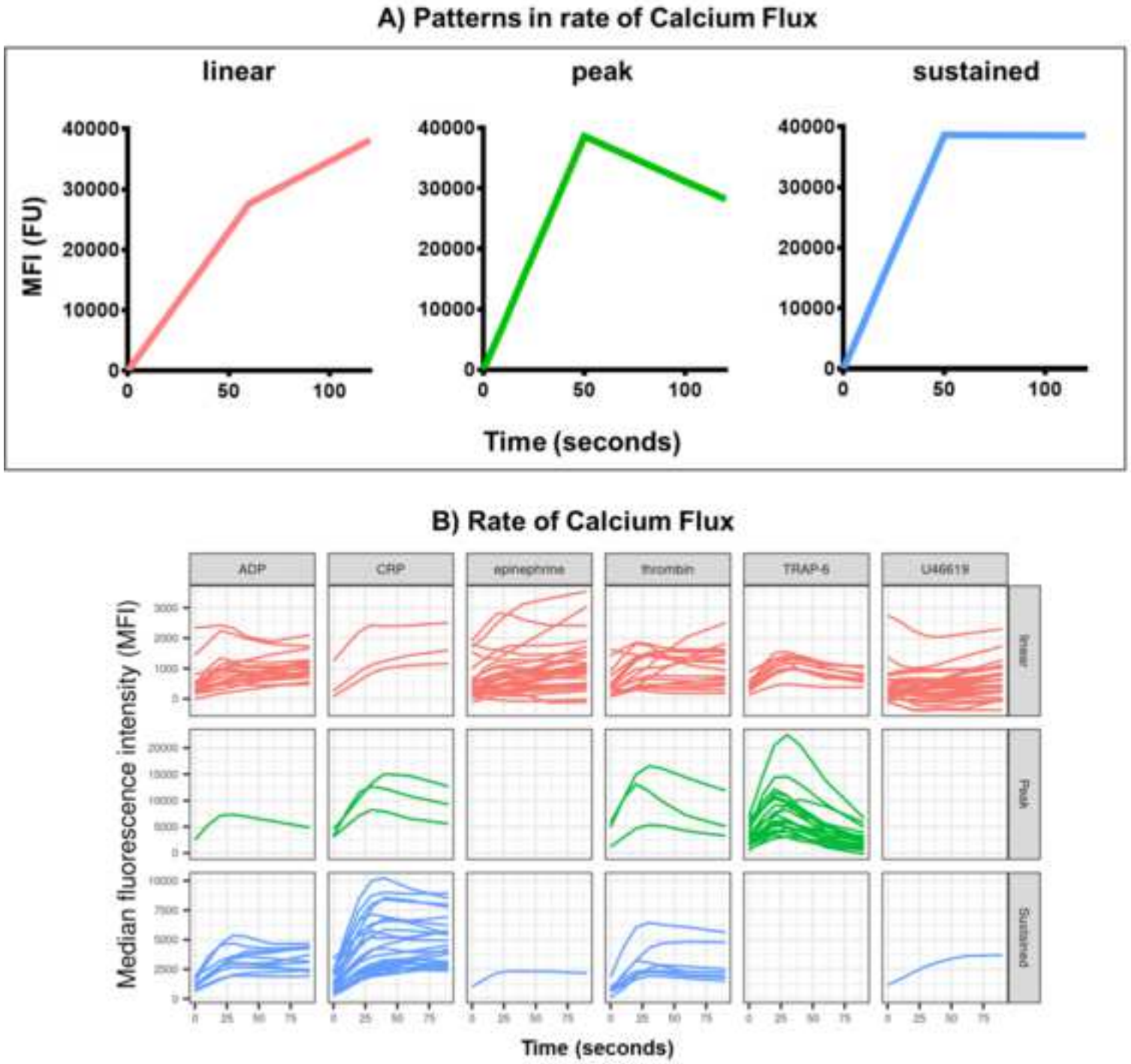
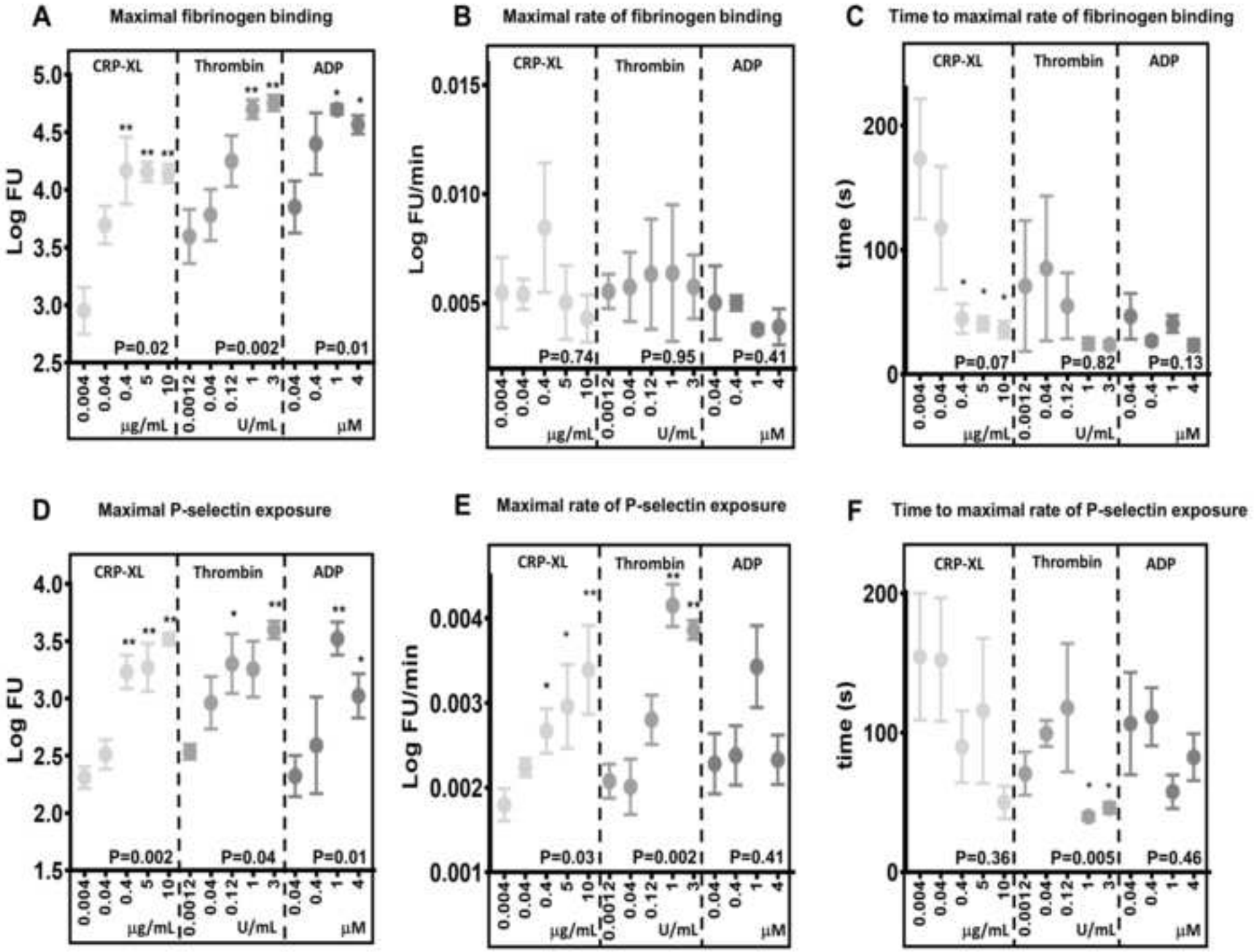


Figure 5



We thank the reviewers for their insightful comments and their constructive feedback and have amended the manuscript accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The new method reported by Dunster et al described a flow cytometry assay to monitor platelet activation in real-time. This protocol provided an efficient kinetic measurement of platelet granule release, fibrinogen binding and intracellular calcium flux. And this is useful to dissect new molecular mechanism during platelet activation. However, there are several points needed to be clearly described.

Major Concerns:

None.

Minor Concerns:

1. The authors should fix the blurred images.

We are sorry for the poor-quality images and have now addressed this.

2. Since this method is dependent on the flow cytometry, the steps of how to set up the parameters should be described in detail.

We agree that there should have been more detail on the set up and have amended the manuscript to include more information on the flow cytometry parameters used.

Reviewer #2:

This paper studies the potential relationship between the rapid response of platelets to vascular injury and extracellular stimuli. It points out that the rate at which platelets convert extracellular stimuli into a functional response is an essential factor in determining how efficiently they can respond to injury, bind to a forming thrombus and signal to recruit other platelets. The author develops a combined flow cytometry assay and analysis software framework to quantitatively measure and categorize platelet activation in real-time. Here are some comments for the authors:

1.The author claims that they have developed a bespoke open-source software Kinetx, which seems to provide feasibility for real-time platelet activation metrics detection. Please add the principle of the Kinetx somewhere in the manuscript.

We are sorry that this was not clear in the original manuscript. The principle of the curve fitting and a description of how the metrics are calculated with in the software are now more obvious within sections 5.2.3.5 and 5.2.3.6 of the protocol.

2.The quality of the figures are low, please update them.

We are sorry for the poor-quality images and have now addressed this.

3.The writing of the manuscript should be improved:
a. The background of the abstract is too long. The authors should use more words to talk about the highlights or achievements of this work.

The reviewer is absolutely right and we have now amended the abstract

b. Calcium flux is a key role in the article, but it is not mentioned in either the introduction or the discussion.

We thank the reviewer for pointing out this oversight and have added sections on calcium in both the introduction and the discussion.

c. Line 297-318. The order of the description of the text and the figure should be consistent.

We apologise for the confusion; the figures are stacked with the fibrinogen plots on top and corresponding P-selectin plots underneath which is why they are not discussed in order as the results are explained from left to right with fibrinogen binding and P-selectin exposure being discussed at the same time.

d. Line 414-435. The figure is not mentioned in the text.

We apologise for missing the figure references out and have added them to the text.

Reviewer #3:

General comments

* The authors preset a method for quantitatively assaying platelet activation endpoints in real time by flow cytometry. The approach is interesting. However: i) several technical points deserve to be defined in a more precise way and ii) already published works deserve to be mentioned and commented (e.g. PMID 10213195; 20872887; 32338820).

Major comments

L127 :

* Was the concentration of the PRP variable or adjusted at a fixed concentration for the experiments?

The PRP concentration was variable with donor platelet counts, platelet concentration was analysed in pilot studies, please see below for an explanation.

* Is the dilution 1:600 correct? (meaning that 0.38 uL of PRP were added to 225 uL of HBS-G-antibody mix)

This dilution is correct, however it is a 1:600 dilution of the final assay volume including the 75 µl agonist so it was added at 0.5 µl in 600 µl. This dilution is explained now in the manuscript with added volumes to ensure clarity.

L144-147 :

* At which final concentration the Fluo-4-AM dye was used?

The dye used was actually the Fluo-4 Direct™ calcium assay from thermofisher and not the FLUO4-AM dye. We apologise for the mistake and have amended this in the manuscript. The dye was used at 1X concentration.

* Was the PRP concentration adjusted for the experiments?

The PRP concentration was not adjusted for the experiments and was added as whole PRP from each donor. We performed pilot studies to examine whether platelet concentration had an effect on rate (both in terms of donor platelet count and dilution in the assay) and it had no effect, we have added this point to the manuscript.

L144-146 :
* Please clarify how the Fluo-4-AM was prepared.

The dye was prepared as described below, we have added this preparation step to the manuscript.

100 mL of Fluo-4 Direct™ calcium assay buffer and 2 mL 250 mM probenecid stock solution was added to one bottle Fluo-4 Direct™ calcium reagent to obtain 2X Fluo-4 Direct™ calcium reagent loading solution. This solution was then mixed 1:1 with HBS-G to achieve a 1X solution and filtered through a 0.22 μM filter prior to use.

* Please specify whether which Fluo-4-AM has been used: the powder or the liquid form in DMSO?

A FLUO4 direct calcium assay kit was utilised (Thermofisher Cat. no. F10472). The Fluo4 was in liquid form and its preparation is detailed in the manuscript.

L153 :
* How do you avoid a continuous loading of the Fluo-4 in the cell when diluting the PRP-Fluo-4/HBS-G in Fluo-4/HBG?

We apologise for the confusion in the original manuscript the platelets are loaded with Fluo-4 Direct, which is designed so that cells are not washed prior to stimulation. Given this it is important we maintain the concentration of Flour-4 direct through out the experiment this is why we keep an equivalent concentration of Flou-4 direct to the initial buffer and in the agonist solution.

* How do you justify to keep adding Fluo-4 in the buffer (3.2.e.) rather than removing it after the first loading (3.2.c.)?

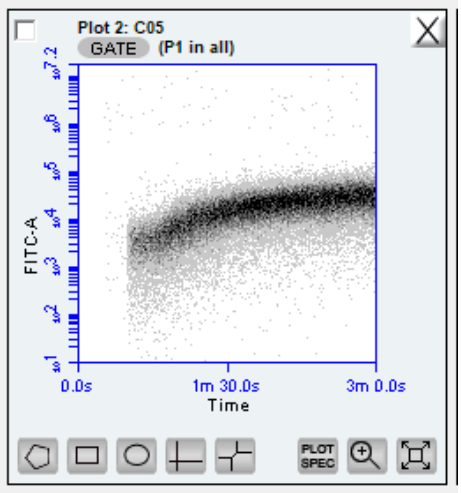
Again we apologies for the confusion in the original manuscript the platelets are loaded with Fluo-4 Direct, which is designed to be used without washing the cells prior to stimulation.

L158 :
* How do you ensure that a 5 sec baseline is stable?

The assay was performed multiple times in its development with longer periods initially and we determined that the sample had consistently stabilised by around 3 seconds so we chose a 5 second timepoint to ensure stabilisation had occurred before the agonist was added.
* Would it not be preferable to record the baseline for a longer period?
L159 :
* How do you check that the "spontaneous mixing" is effective?
* Does it lead to a quick homogenous response?

* Is it possible that the response with different profile rate that were measured were due to an inhomogeneous activation of platelets in the bulk following activation?

The platelet concentration in the final sample was kept very low (1:600) to avoid this from happening. The rapid injection of a volume of 75 µl to 225 µl was sufficient to ensure the sample was immediately well-mixed. This can be observed in the data output on the accuri software which plots the response of each platelet in a smooth curve (example pasted below) which would not occur if only a small proportion of platelets had been activated. We have added a clarification to the manuscript.



L161 :
* What flow speed was used and in average how many events per seconds were collected?
L211 :

The flow speed was always set to low to avoid any platelet clumping and events per second were at an average of 1000 eps and a maximum of 3000 eps. We have added this information to the manuscript.

* How the ranges were established?

Data analysis was commenced at 2.8 seconds to remove variability of observations due to turbulence in the initial seconds after agonist injection. Out of range signals (< 1 or > 2000) were removed to reduce noise for the smoothing algorithm. Because this is open source software individual users can optimise these parameters for there own experimental condition if needed. We have clarified this in the manuscript.

* How was the <2.8 sec limit defined knowing that the Accuri C6 has a delay of 14sec to record events?

The 14 second delay was not included in our methods as we started the timer after this period, we have made this more clear in the manuscript. The 2.8 second limit comes after this 14 seconds.

L365 :
* General comment for the discussion: Better discuss the assay approach and the experimental

observations compared with other published flow cytometric assays (e.g. PMID 10213195; 20872887; 32338820)

We apologise for the oversight and agree that these assays should be discussed and compared, we have now added this to the discussion.

L365 :
General comment. Better discuss the limitations of your assay, namely that the fact that R is not a user-friendly program and compare it to other kinetic modules present in FlowJo, FCSExpress, FACSkin, Flowlogic.

Kinetx was designed to be Open Source, reproducible and easy to implement, in order to get around problems with proprietary software, such as cost and inflexibility. As such it needed to be developed with software that was non-propriety. R was chosen as it is widely used by biologists, easy to install and Open Source. This Open Source environment allows users proficient in R to alter parameters such as degree of smoothing, identification of outlying data points or time scales. However, to aid researchers who are unfamiliar with R, Kinetix was also developed so that analysis can be performed via a single command (either kinetxProcess or kinetxProcessCalcium depending on the data being analysed), and we have tested this accordingly. We hope that by publishing the framework in Jove we can demonstrate how easy the software is to use and encourage others to develop flexible, user friendly open source analysis packages. We have amended the manuscript to address these points.

Figure 3 : As shown on Figure 3, and based on the number of low responder profiles (especially with high dose of thrombin 1U/mL), can you please specify the characteristics of the donors in the methods. How did you define them as "healthy"?

We recruited donors who had no known underlying health conditions, these donors all confirmed they were not taking any medication known to interfere with platelet function. This has now been clarified in the text.

Minor comments
L101-102 :
* Is there any resting time for the blood after punction and before centrifugation?

Blood was rested for 30 minutes prior to centrifugation at 30°C. This information has been added to the manuscript.
* Same question for PRP after centrifugation: at RT or 37°C ?

PRP was centrifuged at room temperature and then PRP was incubated at 37°C after it collection until all samples in the assay had been run which occurred within 1 hour of centrifugation, we have made this more clear in the manuscript.

L110-111 :
* Rephrasing point 3 because the set-up is not clear

We apologise for the lack of clarity here and have rephrased this point.

L152 :
* The brackets are not present for the concentration of the TRAP-6

We have now added these brackets into the manuscript.

L162 :
* It would be handy to specify/repeat what is measured in which assay and which channel at this point

We have now specified what is measured in each channel in the text here.
L262 :
Mistyping CRLXL (should be CRP-XL)

We apologise for this grammatical error and have now amended this in the manuscript.