Pre-analytical conditions for multiparameter platelet flow cytometry.

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ABSTRACT

Background. Flow cytometry is an important technique for understanding multiple aspects of blood platelet biology. Despite the widespread use of the platform for assessing platelet function, the optimisation and careful consideration of pre-analytical conditions, sample processing techniques and data analysis strategies should be regularly assessed. When set up and designed with optimal conditions it can ensure the acquisition of robust and reproducible flow cytometry data. However, these parameters are rarely described despite their importance.

Objectives. We aimed to characterise the effects of several pre-analytical variables on the analysis of blood platelets by multiparameter fluorescent flow cytometry.

Methods. We assessed anticoagulant choice, sample material, sample processing and storage times on four distinct and commonly used markers of platelet activation including fibrinogen binding, expression of CD62P and CD42b, and phosphatidylserine exposure.

Results. The use of sub-optimal conditions led to increases in basal platelet activity and reduced sensitivities to stimulation, however the use of optimal conditions protected the platelets from artefactual stimulation and preserved basal activity and sensitivity to activation.

Summary. The optimal pre-analytical conditions identified here for the measurement of platelet phenotype by flow cytometry suggests a framework for future development of multiparameter platelet assays for high quality datasets and advanced analysis.

KEYWORDS

flow cytometry, blood platelets, pre-analytical, multiparameter

ESSENTIALS

-Flow cytometry is an excellent technique for measuring blood platelet function

-Quality multiparameter platelet flow cytometry requires optimised pre-analytical conditions

-Several pre-analytical conditions were compared, and the best were determined

-Using optimal conditions, robust blood platelet flow cytometry can be performed

INTRODUCTION

The use of fluorescent flow cytometry (FFC) for single cell analysis is standard in many research settings [1,2], including blood platelet biology. The increased availability of multiparameter FFC, spectral FFC, and cytometry by time-of-flight (CyTOF) has driven a significant rise in the number of parameters that can be measured simultaneously. Accordingly there has been a parallel expansion and development of multidimensional analytical tools such as t-stochastic neighbourhood embedded (tSNE) [3], spanning-tree progression analysis of density-normalised events (SPADE) [4] and flow self-organising maps (flowSOM) [5]. Concurrent to advances in the wider field of single-cell analysis, platelet flow cytometry has seen various innovations including multidimensional FFC analysis [6-8], phosphoflow and barcoding [9], application of spectral FFC [10] and CyTOF with multidimensional analysis [11-13]. While the latter may show comparable efficacy to FFC [14] and the design of CyTOF assays has been recently described in full [15], it remains relatively inaccessible when compared with FFC, which is common in research and medical institutes worldwide. The continued use of FFC to analyse platelet function both for basic research and clinical use has led to a recent call by the ISTH SSC on platelet physiology for further studies to facilitate the continued standardisation of platelet FFC protocols [16].

We previously designed a multiparameter FFC panel to measure multiple and diverse facets of platelet function to enable the identification of platelet subpopulations in whole blood [6]. We used the same panel in this study to measure (1) fibrinogen binding as an indirect measure of integrin αIIbβ3 activity, (2) P-selectin (CD62P) expression as a marker of α-granule secretion that is ideal for assessing spontaneous activation since its expression is irreversible *in vitro* [17], (3) annexin V binding as a marker of phosphatidylserine (PS) exposure which is representative of platelet procoagulant activity [18,19], and (4) CD42b (GPIb) to identify *bona fide* platelets in the sample as a component of the constitutively expressed GPIb-IX-V complex. CD42b was also considered an additional marker of activation since it may be shed and/or internalised on activation [20-22]. These markers are ideal for assessing the impact of pre-analytical conditions as they cover a wide-range of key platelet functions, which are reviewed in detail elsewhere [23]. In order to comment on the effects of the pre-analytical conditions we require robust exposure and expression of all markers analysed. In these experiments we used a combination of agonists which we have previously used to induce platelet activation [6].

Beyond assay design and data analysis, which are covered in greater detail elsewhere by general flow cytometry guidelines [1], the optimisation of pre-analytical conditions is unique to the samples under investigation [24]. Here we consider the impact of pre-analytical conditions on platelet phenotype and function. As platelets are primary cells which are collected as whole blood and require donation on the day of experimental analysis, some pre-analytical conditions are especially relevant. These include anticoagulant choice, speed of sample processing, biological sample type, and sample storage. The impact of these four conditions were compared using our four-parameter FFC assay, and optimal conditions are recommended based on the results. These optimal conditions were then examined for subpopulation analysis by opt-FIt-SNE and Phenograph. The recommendations of this study are intended for researchers aiming to utilise FFC to measure platelet function through the expression of one or more markers and to facilitate identification of blood platelet subsets.

METHODS

Flow cytometry. Samples were run on a Beckman Coulter CytoFLEX RUO Flow Cytometer with two lasers (488 nm 50 mW and 638 nm 50 mW) and four detectors (525/40 BP, 585/42 BP, 660/10 BP and 712/25 BP). Avalanche photodiode detector gains were optimised with a LED pulser (quantiFlash, APE) [25]. Automatic compensation was performed with VersaComp Antibody Capture Beads and CytExpert (v2.1).

For sample preparation, whole blood, platelet rich plasma (PRP), and washed platelets (WP) were supplemented with calcium (1.8 mM) and Gly-Pro-Arg-Pro (GPRP: 500 µM) to facilitate Annexin V binding [19] and prevent fibrin polymerisation respectively [26]. Isolation of PRP and WP is described within supplementary information. Samples were incubated with agonists and antibodies for 20 min before fixation with paraformaldehyde solution (0.9% final v/v) [27]. The combination of two agonists for protease-activated receptor-1 and GPVI, the peptide agonist SFLLRN (20 µM) and CRP-XL (10 µg/mL) respectively, were used to activate platelets and induce PS exposure [8]. Ten thousand genuine platelet events were recorded based on CD42b expression (Fig. S1), and doublet events were excluded as previously described [6]. We validated CD42b as a genuine platelet marker (≥99%) by comparing it with CD41 and CD36 expression (Fig. S2). Positive gates were set with perfectly matched isotype controls (IgG-PE, CD62P) and internal negative controls (ethylenediaminetetraacetic acid (EDTA) 10 mM, anti-fibrinogen/Annexin V). Antibodies were used at optimal titres (Table S1), and assays were designed in accordance with established guidelines [23,28-31].

Data analysis. Flow cytometry standard (FCS) files were analysed on CytExpert (v2.1, Beckman Coulter) and FlowJo (v10.7.1, BD Biosciences), and statistical analysis was performed by ordinary one-way ANOVA with multiple comparisons (Šídák's) on GraphPad Prism (v8.0.0). Statistical significance was accepted as * $p<0.05$, ** $p<0.01$, *** $p<0.005$ and **** $p<0.001$. CytExpert was used to export compensated MFI and percentage positive data while FlowJo was used to present concatenated FCS data as stacked histograms. Median fluorescence intensities (FI) were used for all markers except annexin V, which was expressed as the mean FI. In addition to mean±SD, we also included individual data points. Results were reported as both percent positive and MFI, rather than only presenting a portion of the data acquired. Percent positive is traditional for heterogeneous markers [32] but requires a background control (*e.g.,* fluorescence minus one or isotype control), and lacks relevance when all cells are positive. However, MFI does not require gating controls and can differentiate between low and high levels of expression when all cells are positive (*ie.,* 100%). FlowJo was also used to implement opt-FIt-SNE [33,34] and Phenograph (v3.0) [35]. Opt-FIt-SNE was performed with a perplexity of 30, 1000 iterations and an ANNOY library while Phenograph was performed with 100 nearest neighbours, and data were visualised using ClusterExplorer (v1.6.5). These analyses were performed on CD42b+ events from concatenated files of 3 donors.

See supplementary information for materials, venepuncture and additional methods.

RESULTS

Anticoagulants. Anticoagulants are perhaps the most important consideration for a platelet biologist, as by their design they can significantly alter and affect platelet function. Here we tested platelet phenotype in three commonly used anticoagulants; sodium citrate (BD), sodium heparin (BD) and K_2EDTA (Greiner Bio-One), all of which were commercially prepared in evacuated blood collection tubes [36].

Measuring fibrinogen binding we showed that none of the anticoagulants increased basal binding of fibrinogen to the platelet surface (Fig. 1Aii). However, in response to stimulation (SFLLRN/CRP-XL) the greatest binding of fibrinogen (MFI) was observed in citrated blood followed by heparin with a significant reduction in fluorescence when anticoagulated by EDTA ($p<0.01$) (Fig. 1Ai). The anticoagulant effect of EDTA is primarily through the chelation of calcium, where platelet-fibrinogen interactions are dependent on the availability of free calcium. When CD62P expression was assessed we found that the α-granule marker was elevated on the cell surface in heparin (23.8%±16.8) and EDTA ($p<0.05$, 42%±19.5) anticoagulated blood under basal conditions when compared to citrate (14.5%±6.7, Fig. 1Bii). CD62P expression in response to stimulation (SFLLRN/CRP-XL) was no different between any of the anticoagulants (Fig. 1Bi). Annexin V binding as a marker of procoagulant platelets showed no differences at basal when comparing the anticoagulants, however in heparinised blood this showed the highest fluorescence intensity in response to agonists (SFLLRN/CRP-XL), nevertheless this was not significantly increased over citrate (Fig. 1Ci). The procoagulant platelet subpopulation has been described previously as up to two thirds of the total population of platelets [15]. When procoagulant platelets are measured, the proportion of PS positive cells does not change significantly between the anticoagulants and sits within that range (Fig. 1Cii). As a final measure of activity we recorded the loss of CD42b from the platelet surface, this was shown to be independent of the anticoagulant used at both basal and in the presence of stimuli (SFLLRN/CRP-XL) (Fig. 1D).

Sample processing. To understand the effects of short-term storage of whole blood on platelet function, we compared freshly collected whole blood (citrate anticoagulated) with the same sample stored for 1.5 or 4.5 hours at room temperature [37] and compared functional platelet phenotypes.

Basal fibrinogen binding did not change overtime, however it showed a dramatic reduction in signal in response to stimulation (SFLLRN/CRP-XL) over the time course tested. MFI was significantly lower at 1.5 hr ($p<0.001$), and lowest at 4.5 hr ($p<0.001$) (Fig. 2Ai). This reduction was also observed in percent positive cells, where the positive population decreased over time and was significantly reduced at 4.5 hr ($p<0.05$) (Fig. 2Aii). Delays in sample processing induced significant artefactual activation, as the percentage expression of basal CD62P increased significantly at 1.5 hr ($p<0.01$) and 4.5 hr ($p<0.001$), ultimately resulting in near doubling of baseline expression (Fig. 2Bii). Mirroring fibrinogen binding, capacity for CD62P expression by agonist (SFLLRN/CRP-XL) was also diminished at both 1.5 hr ($p<0.01$) and 4.5 hr ($p<0.05$) (Fig. 2Bi). Annexin V binding was less sensitive to the effect of storage with no significant changes over time (Fig. 2Ci), although there was a trend towards increased basal PS exposure (Fig. 2Cii). We postulate that over a longer period platelet function may continue to decline and basal PS exposure would likely increase [38-40] as PS exposure is also indicative of apoptosis. In support of this observation, mitochondrial membrane potential ($\Delta m\Psi$) decreases over a similar time course (Fig. S3), and a loss of $\Delta m\Psi$ is typically paired with an

increase in PS exposure [6]. The period of storage results in loss of basal CD42b at 1.5 hr ($p<0.05$) and 4.5 hr ($p<0.001$) (Fig. 2D), suggesting spontaneous shedding/and or internalisation. However, CD42b expression remained sensitive to agonist stimulated loss with no difference across the different time points.

Biological samples. Here we compare citrated whole blood, PRP (isolated from citrate anticoagulated blood), and WP (isolated from ACD anticoagulated blood) to determine the impact of sample material on the measurement of functional platelet parameters. All samples were paired to allow for accurate comparisons, *ie.,* whole blood, PRP and WP were all from the same venepuncture from the same donor.

Basal fibrinogen binding was similar in all sample types with a non-significant increase in WP compared with PRP and WB. However, agonist-stimulated (SFLLRN/CRP-XL) fibrinogen binding was similar between whole blood and WP, but fibrinogen binding was diminished in PRP (Fig. 3Ai). On further examination of the percent positive data, WP also lost reactivity compared to whole blood ($p<0.005$). The EDTA-treated control indicates the high MFI in WP is due to an increased background signal, which in turn affects the ability to detect true positive signal (Fig. 3A). This may be a result of the increased binding of fibrinogen to the platelets during isolation, which occurs prior to the EDTA addition. In terms of CD62P expression there was a significant increase in basal expression linked to artefactual activation when measured as percentage. This was high in PRP ($p<0.001$, 35.1±9.1%) and higher still in WP ($p<0.001$, 69.5±12.2%) compared with whole blood (14.5±6.7%) (Fig. 3Bii). Paired with this, CD62P expression in both isolated samples showed a non-significant reduction in agonist (SFLLRN/CRP-XL) driven expression when compared to whole blood (Fig. 3Bi). Annexin V binding at basal was increased in both PRP and WP (Fig. 3Ci), with a significantly greater proportion of platelets positive for the marker when stimulated (Fig. 3Cii). It has been previously shown (in whole blood) that the number of PS positive cells does not change based on agonist availability [8] suggesting the increased PS exposure may be due to increased artefactual pre-activation of these cells, driving hypersensitivity to stimulation. CD42b binding data mirrored CD62P expression and showed a loss of signal in basal samples, mimicking the increased basal activity, in both PRP ($p<0.005$) and WP ($p<0.001$) while sensitivity to agonist (SFLLRN/CRP-XL) stimulation was maintained (Fig. 3D).

Storage. Here we stained and acquired citrated whole blood samples immediately after fixation, and then after storage (at 4°C in the dark) for 1.5 hr and 4.5 hr the samples were repeatedly acquired for a second and then third time accordingly. We found that measures of fibrinogen binding remained robustly stable with no change in MFI (Fig. 4A). However, there was a significant reduction ($p<0.001$) in detection of agonist-stimulated CD62P expression at both 1.5 and 4.5 hr (Fig. 4B) mirrored by a significant reduction ($p<0.001$) in detection of agonist-stimulated annexin V binding at 1.5 and 4.5 hr (Fig. 4C). CD42b expression was not significantly altered over the storage times tested (Fig. 4D).

Multidimensional analysis. Using our optimal conditions described in the previous sections, we performed Opt-FIt-SNE [33,34] and Phenograph [35] clustering on concatenated datasets from replicate experiments. Here we analyse both basal and stimulated samples, which allows for a direct comparison of the changes to the phenotypic subsets induced by robust dual-agonist (SFLLRN/CRP-XL) driven platelet activation. Combining these two techniques allows the projection of 4D data onto a 2D tSNE map (Fig. 5a), which is false coloured by the Phenograph clusters (Fig. 5b). While similar numbers of clusters are identified when comparing basal and

stimulated, the subsets are more distinct when the samples have been stimulated. The tSNE map is heat-scaled for expression of each of the four parameters and shows both high (warm) and low (cool) zones of expression across the population (Fig. 5c). Many of these zones of high and low expression are also identified as distinct subpopulations by Phenograph, which suggests these tools are clustering and identifying analogous subsets. The clusters identified across the platelet populations by Phenograph can also be compared for relative expression of each marker using a heat map, allowing interrogation of the marker expression profile and proportion of each individual subpopulation (Fig. 5d). Together these data demonstrate the almost universally low expression of activation markers on a basal sample, contrasted with the predominantly high but differential expression in stimulated samples (Fig. 5d-e). Finally, it is possible to retrospectively project these Phenograph subpopulations onto the tSNE map (Fig. 5e), highlighting clusters by their positivity for each individual marker, their unique fingerprint.

DISCUSSION

In the following sections we describe our pre-analytical; anticoagulant, sample processing time, biological sample material and sample storage recommendations for development of multiparameter platelet function FFC assays. We present data from samples which were simultaneously probed for four markers and from this we assess the impact of different conditions on each marker individually. Finally, we investigate the optimum conditions with advanced analytical techniques.

Anticoagulants. Based on our findings here we suggest that citrate is the optimal anticoagulant for measuring multiple aspects of platelet function in the same assay sample. Citrate gave the greatest sensitivity to stimulation, reflected in the highest level of agonist-induced fibrinogen binding and the lowest level of basal activity demonstrated by CD62P expression compared to the other anticoagulants tested. Furthermore, citrate did not impede the ability to detect procoagulant platelets, while staining of CD42b was consistent with other anticoagulants. Our findings also corroborate the observations made by others, while providing additional information by measuring markers of platelet function which have not been previously characterised in this way. Citrate, heparin and hirudin anticoagulants were previously assessed for their effects on PAC1 (activated integrin αIIbβ3), CD62P, and LAMP1 [41,42] and in agreement with our study, citrate tubes were considered optimal. Another study comparing citrate, EDTA, heparin and several coagulation factor inhibitors measuring only CD62P identified that citrate allowed a greater degree of platelet activation [43]. Additionally, EDTA and heparin can drive cell swelling and artefactual activation [41,44,45], and EDTA maintains platelets in a state of hyporeactivity [46]. The potent inhibitory effects of EDTA are likely driven by the high-binding constant of metal ions when compared to a milder chelator like citrate [47,48], where metal ions, in particular calcium, are vital for platelet activation [49]. In lieu of the availability of citrate anticoagulated blood, heparin anticoagulated would be suggested as the second-line choice.

Sample processing. During storage after blood collection, platelets begin to lose reactivity and become basally activated [44]. This challenge has led to a stringent set of conditions for storage of isolated platelets in a clinical setting. While these conditions can allow for several days of storage, even under these optimal conditions reactivity wanes [39,41] and the platelet

storage lesion develops [50]. We believe that the time-frames used here mimic delays which could be experienced by platelet researchers waiting for receipt of samples.

The significant fall in fibrinogen binding did not directly mirror the smaller reductions observed in CD62P and CD42b expression. This may suggest a compounding factor beyond a loss of platelet reactivity during storage is involved. Work from others suggests loss or inactivity of integrin $\alpha_{IIb}\beta3$ is unlikely to account for this as PAC1 binding does not decrease over time [51] nor does fibrinogen degrade during storage at ambient temperature [52]. GPRP addition is a possible reason for the loss of fibrinogen binding, as it may be incompatible with samples that are stored for extended times [53]. As an alternative to fibrinogen binding, PAC1 may be used to detect the activation of integrin αIIbβ3 [54]. While the use of PAC1 may alleviate loss of fibrinogen signal, there would still be a time dependent decrease in reactivity, as demonstrated by other markers measured here. While it is currently considered appropriate to rest samples for up to 30 minutes before *ex vivo* activation [16], we would recommend that sample preparation occurs as soon as possible after venepuncture. These data suggest there is increased basal activity paired with diminished reactivity as storage time increases. Where delays in sample processing are unavoidable, they should be standardised and increases in basal platelet activity paired with diminished reactivity should be anticipated and appreciated.

Biological samples. Platelet flow cytometry is commonly performed in whole blood samples, but PRP and WP can also be used. Sample material appears to have a significant impact on platelet reactivity and/or increases in basal expression. It is the process of isolation, manipulation, centrifugation, time and buffers required to isolate platelets from the sample which likely affects platelet function.

The sample material is an important choice when designing experimental protocols for primary cells. Our data here suggests that for analysis of platelet function whole blood is optimal as it allows the greatest response to agonist stimulation and the lowest levels of artefactual activation. The use of isolated platelets in the forms of PRP or WP led to increased basal activation of markers, likely a combinatory effect of isolation time, physical manipulation (pipette & centrifuge) and buffer changes. However, as we previously examined the effects of time alone, which is different to data presented here, this suggests that manipulation and buffer changes play the main role in affecting platelet function compared to time alone. The significant increase in basal activity means that percent positive cells loses utility, as basal signal may no longer truly represent the activation state of platelets *in vivo.* This is an issue where clinical models are used, as changes in basal activity are often a key observation. However, the use of PRP or WP to measure *in vitro* sensitivity to agonists/inhibitors remains a viable option, or indeed a necessity in instances where reagents may not be compatible with whole blood. Ultimately where high-sensitivity assays are required to comment on basal activation status of platelets, particularly in clinical samples, or to identify platelet subpopulations, whole blood should be the biological sample of choice.

Storage. Stable storage of prepared samples is an important consideration in flow cytometry assays, particularly in settings with core facilities where machine load determines availability. Having determined previously that citrated whole blood is the optimal approach for these experiments we focussed on this particular condition to understand the effects of short-term fixed sample storage.

While fibrinogen binding and CD42b remained robust, CD62P expression and annexin V binding were diminished by storage. Other studies have shown that as a marker CD62P is

stable up to 5 days [55]; however this is highly dependent on both the fluorophore-conjugate [56] and storage buffer, as formaldehyde can induce fluorophore quenching [57]. For longer term storage it may become prudent to wash the cells out of fixative and resuspend in PBS. However, it is important to note that the decrease in MFI is not reflected by a decrease in the number of cells staining positive for these markers (data not shown), meaning quantitative value is retained in these samples. We recommend that samples be acquired as soon as possible, unless a standardised storage time is used to minimise errors among data that is acquired at different time points.

Multidimensional analysis. Software for multidimensional analysis are being increasingly used in the field of platelet biology, with multiple functions now recognised beyond conventional haemostasis/thrombosis [58]. Opt-FIt-SNE is a method of dimensionality reduction, where events with similar characteristics are plotted in 2-dimensional (2D) space for the robust and semi-automated visualisation of subsets of related cells. Phenograph clusters cells based on shared characteristics and identifies subpopulations with common phenotypic signatures. Performing the two analyses together enriches the discovery of any subsets within the data.

In this example, most cells (95.35%) are CD62P positive whereas only a small number of cells (4.65%) are low for expression of all markers. The data is more interesting when comparing fibrinogen positive (70.17%) and PS positive (28.1%) cells as they are mutually exclusive in clusters 2, 4 and 15, but are both expressed in cluster 8, which reinforces recent observations by ourselves and others [6,10,59,60]. Ultimately, this is a limited example of both the analytical tools and applications available, but as multiparameter FFC panels develop in complexity the data generated by these methods will become exponentially richer and provide greater novelty and discovery.

SUMMARY

We have compared several pre-analytical factors relevant to multiparameter platelet flow cytometry using a panel of markers which cover several aspects of platelet function (Table S2). In the context of this marker-fluorophore combination, the recommendation is that blood is drawn into tubes anticoagulated with citrate, used with minimal manipulation and that samples are prepared and acquired immediately, unless these steps can be standardised. If data is obtained with high confidence, it can then be examined by advanced analytical tools and used to explore and discover novel platelet phenotypes and subpopulations.

AUTHOR CONTRIBUTIONS

MSH designed the research, performed experiments, analysed the data and wrote the manuscript. LTC isolated platelets. DMY performed supplementary experiments. KMN supervised the research and edited the manuscript.

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DECLARATION OF INTERESTS

We declare no competing interests.

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FIGURE LEGENDS

Figure 1. Effects of different anticoagulants on platelet activation. Whole blood drawn into sodium citrate, sodium heparin and potassium EDTA vacutainers were compared for multiparameter platelet activation at basal or stimulated with SFLLRN (20 µM) alone and with CRP-XL (10 µg/mL). Stimulated EDTA and isotype controls are included for reference to assay performance. (A) Fibrinogen binding, (B) CD62P expression, (C) PS exposure, (D) CD42b expression, (i) indicates MFI data and (ii) percentage positive data. (n=3±SD)

Figure 2. The impact of sample processing time on platelet activity. Multiparameter platelet activation was assayed in whole blood drawn into sodium citrate at 0 hours (freshly drawn), 1.5 and 4.5 hr post-draw at basal or stimulated with SFLLRN (20 µM) alone and with CRP-XL (10 µg/mL). (A) Fibrinogen binding, (B) CD62P expression, (C) PS exposure, (D) CD42b expression, (i) indicates MFI data and (ii) percentage positive data. (n=4±SD)

Figure 3. The sample material impacts assay sensitivity. Whole blood was compared with PRP and WP for multiparameter platelet activation, at basal or stimulated with SFLLRN (20 µM) alone and with CRP-XL (10 µg/mL). Stimulated EDTA and IgG controls are included for reference to assay performance. (A) Fibrinogen binding, (B) CD62P expression, (C) PS exposure, (D) CD42b expression, (i) indicates MFI data and (ii) percentage positive data. (n=3±SD)

Figure 4. Effect of sample storage on assay performance. Samples fixed and ran immediately compared with 1.5 and 4.5 hr 4^{o}C dark storage were compared for multiparameter platelet activation, at basal or stimulated with SFLLRN (20 µM) with CRP-XL (10 µg/mL). (A) Fibrinogen binding, (B) CD62P expression, (C) PS exposure, (D) CD42b expression, (i) indicates MFI data and (ii) percentage positive data. (n=4±SD)

Figure 5. opt-FIt-SNE and Phenograph analysis of platelet subpopulations under optimal pre-analytical conditions. 30,000 platelet events from either basal samples or samples treated with SFLLRN (20 µM) and CRP-XL (10 µg/mL) were concatenated and analysed by opt-FIt-SNE and Phenograph then visualised by ClusterExplorer. (A) tSNE map false coloured for Phenograph clusters described in (B). (C) tSNE map false heat-coloured (hot-cold/high-low) for indicated marker expression. (D) Heat map (hot-cold/high-low expression) for each cluster identified by Phenograph and relative marker expression within each subpopulation. (E) Stimulated sample only tSNE map highlighted with clusters positive for each marker.

Table 1. Summary of recommended pre-analytical conditions. Having compared the effects of anticoagulants, sample processing conditions, biological sample material and sample storage on platelet function, we have described the optimal condition for platelet flow cytometry for each of these situations.

Table one. Recommended conditions.

Pre-analytical condition	**Optimal condition**
Optimal anticoagulant	Sodium citrate
Sample processing	Process as soon as possible
Biological sample	Whole blood
Sample storage	Avoid or standardise storage

Figure one. Anti-coagulant

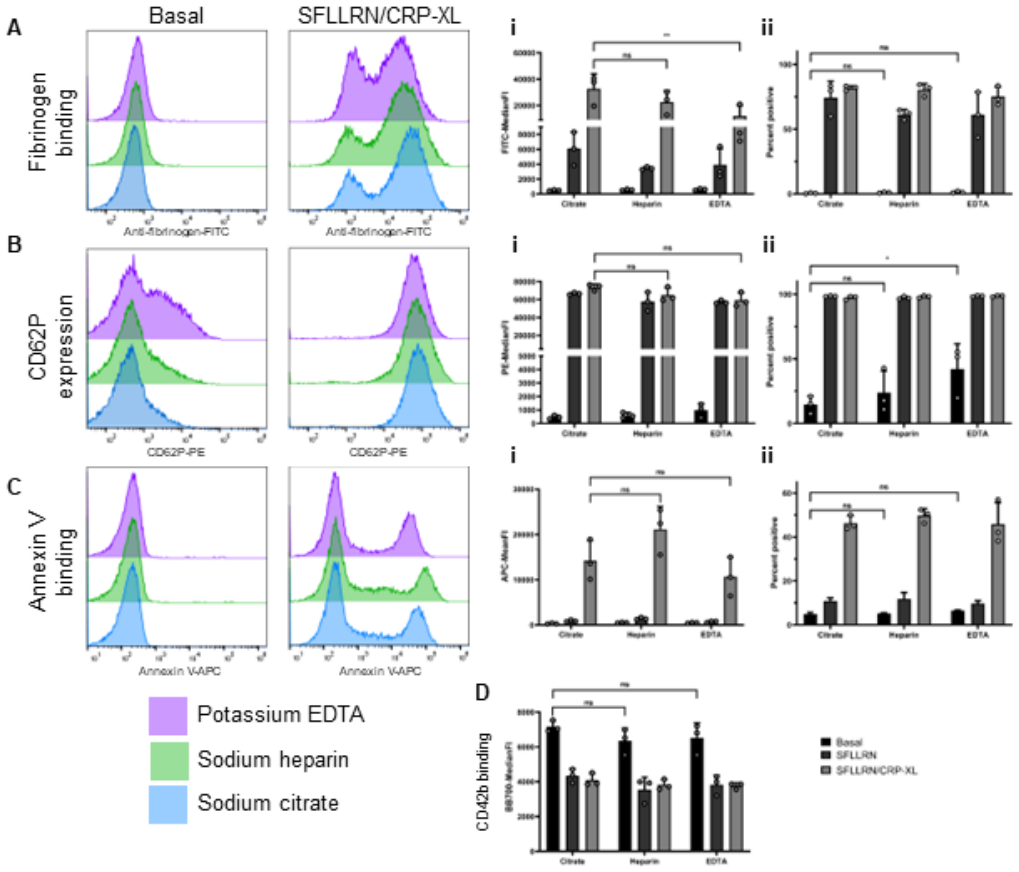

Figure two. Sample processing time

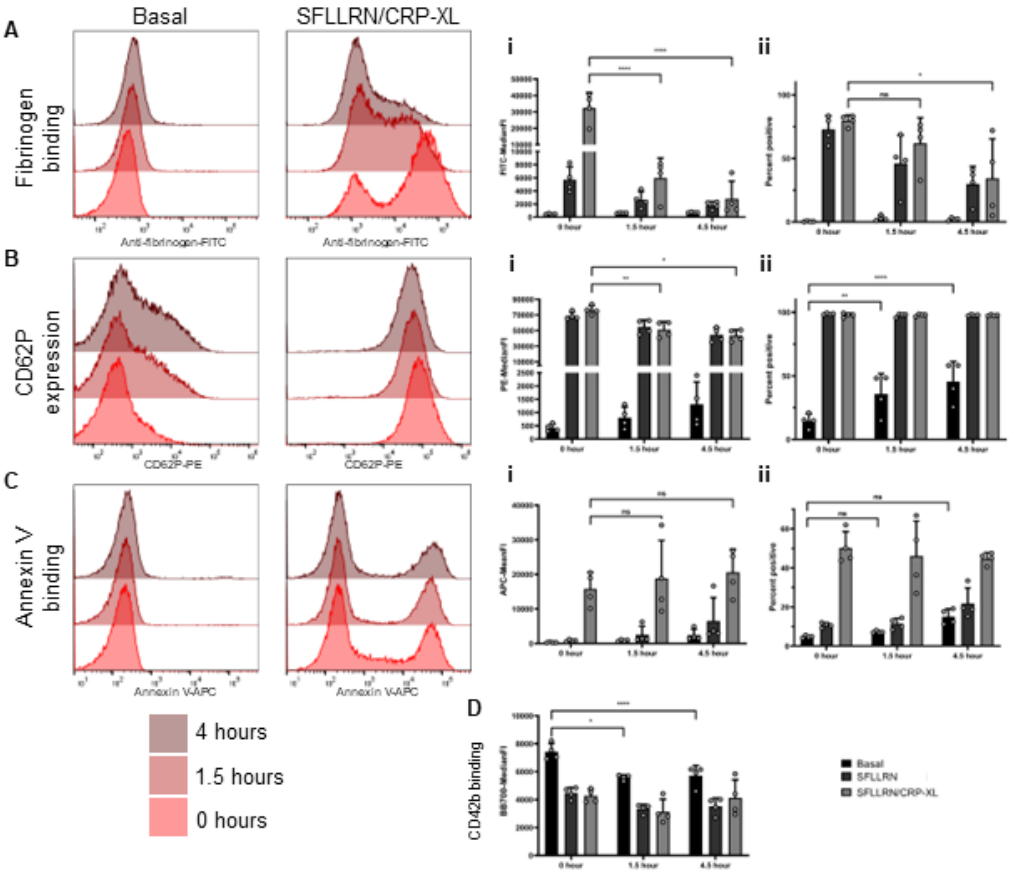

Figure three. Sample material

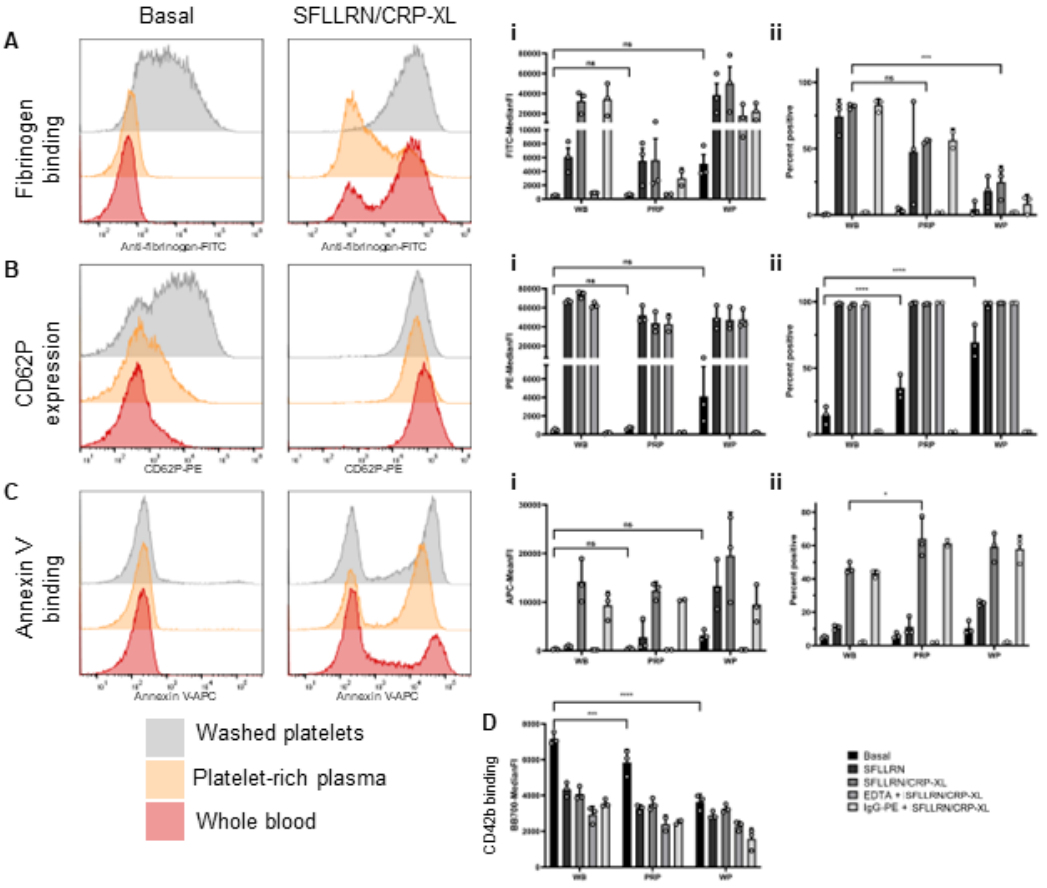

Figure four. Sample storage

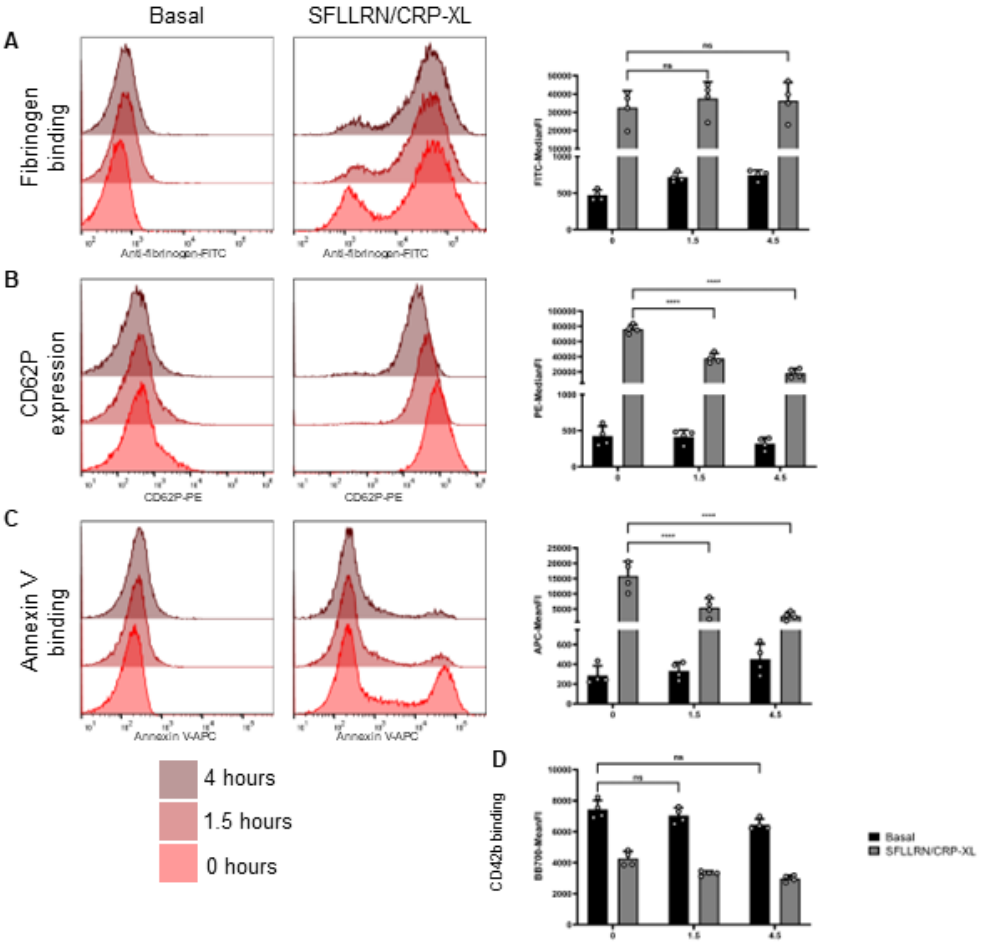

Figure five. Multidimensional analysis