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TECHNICAL REPORT

A multi-color flow cytometric method for characterizing murine reticulated platelets using SYTO 13

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Abstract

Reticulated platelets (RP) are immature platelets with heightened RNA content. An increased level of RP in the circulation is associated with various pathological conditions. In this study, we employed a novel flow cytometry approach for RP detection in mice, utilizing the nucleic acid dye SYTO 13 in conjunction with a platelet-specific marker (anti-mouse CD42b-DyLight 649). The efficacy of SYTO 13 for RP identification was confirmed by higher circulating RP levels in platelet-depleted mice during the recovery phase (35% ± 13%) compared to untreated mice (11% ± 1%, $n = 9$, $p < .0001$). We further characterized murine RP by exploring the surface expression of the platelet activation marker P-selectin. While there was no preactivation at baseline, stimulation with a thrombin receptor agonist (PAR-4) resulted in a higher increase in the percentage of P-selectin-positive platelets in RP (93% ± 6%) compared to non-RP (77% ± 14%, $n = 6$, $p = .0065$). In addition, RP exhibited higher geometric mean fluorescence intensity levels than non-RP (1874 ± 1278 *versus* 859 ± 549, $n = 6$, $p = .02$). Our proof-of-principle study demonstrates the efficacy of SYTO 13 in combination with platelet (activation) markers for identifying RP in both resting and activated states. This method holds promise for simultaneously monitoring RP levels and platelet activation in murine models of human disease.

Keywords
Flow Cytometry, Reticulated Platelets, Immature Platelets, Platelet Subpopulations, SYTO 13

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Introduction

Reticulated platelets (RP), also known as immature platelets, are the youngest platelets released from megakaryocytes and are characterized by heightened RNA content, and increased activation upon agonist stimulation.[1–3] Due to their enhanced reactivity compared to mature platelets, RP have been implicated in impaired response to antiplatelet therapy.[3] In clinical settings, immature platelet levels are determined using automated hematology analyzers, providing values like immature platelet fraction (IPF%) or immature platelet count (#IPC). While IPF offers clinical insights, it has limitations in facilitating further investigations and is biased in cases of platelet size alterations.[4]

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To overcome these limitations, fluorescence flow cytometry using nucleic acid binding fluorochromes like thiazole orange (TO) is employed.[5] However, TO has drawbacks, including low fluorescence quantum yield and a limited stability over time.[6,7] SYTO 13 is another cell permeable dye and highly green fluorescent when bound to nucleic acids.[8] In human samples, SYTO 13 emerges as a superior alternative, demonstrating prolonged stability over time compared to TO, allowing extended experimental analysis, and exhibiting a higher quantum yield.[7,9] Recognizing the importance of murine models in platelet biology research, we present a novel method for detecting RP in mice using SYTO 13. Our protocol not only detects RP levels but also monitors their activation status and enables simultaneous staining for other markers of interest. This advancement in RP detection, minimizing interference from cell size parameters, holds promise for comprehensive investigations in both clinical and experimental contexts.

Materials and methods

Materials and reagents

A comprehensive list of all materials and reagents used in this study can be found in the supplementary data (Table S1).

Ethical statement

The animal procedures adhered to the German animal protection law and were approved by the federal authorities in Baden-Württemberg, Germany (35–9185.81/G-20/60).

Blood collection

C57BL/6J mice were anesthetized with 100 mg/kg body weight ketamine and 20 mg/kg body weight xylazine. Blood was obtained by terminal cardiac puncture using a 24-gauge needle coated with unfractionated heparin. Blood was anticoagulated with 1.5 µg/µL enoxaparin and diluted 1:10 with Dulbecco′s phosphate buffered saline containing calcium and magnesium (DPBS+/+). All samples were processed within 30 min after blood collection.

Stimulation of RP production

To increase RP levels, acute thrombocytopenia was induced in mice by a single subcutaneous injection of 1 µg/g body weight of a GPIbα (CD42b) targeting antibody (R300, Emfret).[10,11] The successful depletion of platelets was confirmed by analyzing tail vein blood one day after the antibody injection using the automated hematology analyzer Sysmex XN (Sysmex Corporation, Kobe, Japan). Subsequently, on the fourth day following platelet depletion, blood was collected as described above.

Staining protocols

SYTO 13 time course

Diluted blood was incubated with 5 µM SYTO 13 and anti-CD42b-DyLight 649 for varying durations (15, 30, 60, or 90 min) at room temperature (RT). Then, blood was mixed and incubated with prewarmed (37°C) phosflow lyse/fix buffer (15 min, RT, in the dark), centrifuged (700 × g, 5 min, RT), resuspended in DPBS+/+ and analyzed by flow cytometry. An alteration in the protocol involved staining with anti-CD42b-DyLight 649 after fixation (15 min, RT, in the dark) followed by washing with DPBS+/+ (700 × g, 5 min, RT), resuspension in DPBS+/+ and analyzing by flow cytometry.

SYTO 13 titration

Diluted blood was incubated with PBS (0 µM) or different SYTO 13 concentrations (ranging from 1.6–100 µM) for 15 min at RT. Then, blood was mixed and incubated with prewarmed (37°C) phosflow lyse/fix buffer (15 min, RT, in the dark) and centrifuged (700 × g, 5 min, RT). Pellets were resuspended in DPBS+/+ and incubated with anti-CD42b-DyLight 649 (15 min, RT, in the dark). Finally, samples were washed with DPBS+/+ (700 × g, 5 min, RT), resuspended in DPBS+/+ and analyzed by flow cytometry. An alteration in the protocol involved first fixation and then simultaneous incubation with SYTO 13 and anti-CD42b-DyLight 649, with all other steps kept the same.

RP detection in platelet-depleted mice

Diluted blood from mice on day 4 following platelet depletion was mixed and incubated with prewarmed (37°C) phosflow lyse/fix buffer (15 min, RT, in the dark) and centrifuged (700 × g, 5 min, RT). Pellets were resuspended in DPBS+/+ and incubated simultaneously with SYTO 13 (25 µM) and anti-CD42b-DyLight 649 (15 min, RT, in the dark). Finally, samples were washed with DPBS+/+ (700 × g, 5 min, RT), resuspended in DPBS+/+ and analyzed by flow cytometry.

Surface P-selectin expression in RP and non-RP

Diluted blood from naïve mice was incubated with murine PAR-4 agonist (400 µM) or DPBS+/+ (15 min, RT). Then, samples were incubated with anti-CD62P-PE/Cy7 antibody or the corresponding isotype control (15 min, RT, in the dark), mixed and incubated with prewarmed (37°C) phosflow lyse/fix buffer (15 min, RT, in the dark) and centrifuged (700 × g, 5 min, RT). Pellets were resuspended in DPBS+/+ and incubated simultaneously with SYTO 13 (25 µM) and anti-CD42b-DyLight 649 (15 min, RT, in the dark). Finally, samples were washed with DPBS+/+ (700 × g, 5 min, RT), resuspended in DPBS+/+ and analyzed by flow cytometry.

Data analysis and gating

Flow cytometry data was acquired using a FACSCanto II flow cytometer (BD, Franklin Lakes, NJ, USA) with subsequent analysis using FlowJo version 10 (Tree Star, Ashland, OR, USA). The gating strategy initiated with single-cell identification based on forward-scatter (FSC) characteristics (FSC-A and FSC-H). Platelets were then distinguished by sideward-scatter (SSC-A) characteristics and positivity for the platelet-specific marker CD42b. Further identification of platelet subsets was achieved based on SYTO 13 intensity, where high intensity indicated the RP population, and lower intensity represented the non-RP population.

Data are presented as mean values ± standard deviation (SD). Statistical analyses utilized GraphPad Prism version 9. Two-tailed unpaired or paired t-test was performed to compare the means between two groups. Repeated measures one-way analysis of variance with Bonferroni multiple comparison test was used to test for significant differences between more than two groups. A p value < 0.05 was considered statistically significant.

Results

SYTO 13 time-course analysis for RP identification

We initiated the development of the RP detection method for murine platelets by experimenting with various time points for SYTO 13 incubation (15, 30, 60, 90 min), and a fixed SYTO 13 concentration (5 µM) (Figure 1). These conditions were adapted from those employed in human RP detection.[7] Notably, we determined that a 15-min incubation period was adequate, with no significant changes in the percentage of RP even with extended incubation times. However, we noted the possibility of platelet aggregate formation, which could lead to an overestimation of RP percentages (Figure 1A). To address this, we tested adding the platelet labeling antibody (anti-CD42b-DyLight 649) post-fixation, effectively preventing the potential aggregate formation (Figure 1B) and resulting in RP percentages consistent with those typically observed in murine samples.[12] Consequently, we adopted a SYTO 13 incubation time of 15 min and the addition of anti-CD42b-DyLight 649 post-fixation for all subsequent experiments.

SYTO 13 titration analysis for RP identification

Next, we proceeded to evaluate different SYTO 13 concentrations to determine the most suitable one. Initially, we stained unfixed whole blood (SYTO 13 added before fixation), noting a concentration-dependent effect, requiring higher SYTO 13 levels for adequate RP level separation (Figure 2A,C). Higher SYTO 13 concentrations reduced CD42b expression (decreased geometric mean fluorescence intensity, Figure S1A). To enhance staining, we modified the protocol by adding SYTO 13 after

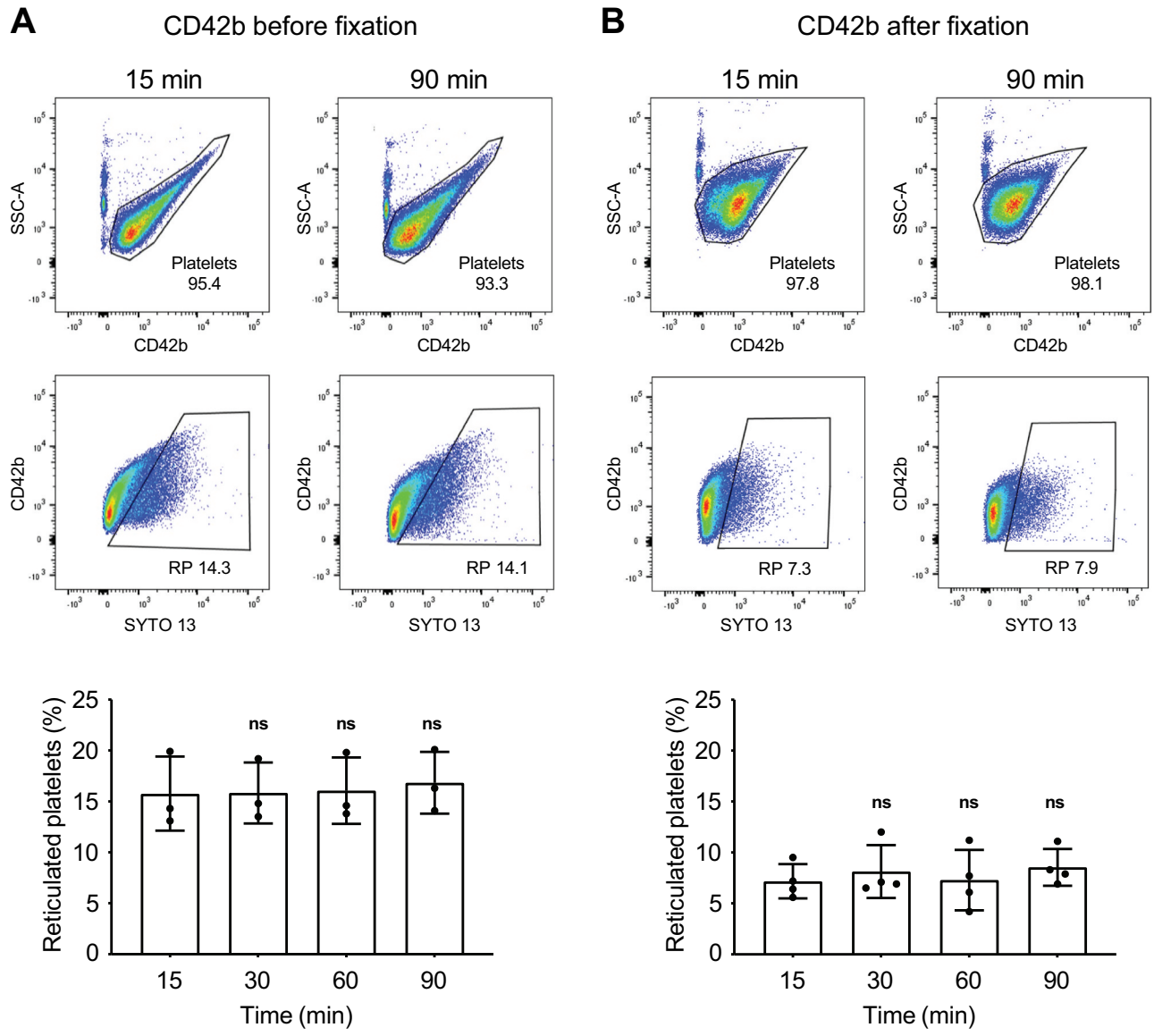

Figure 1. Analysis of reticulated platelets using CD42b staining before or after fixation. The percentage of reticulated platelets was determined at baseline in anticoagulated blood (1:10 diluted) following incubation with SYTO 13 (5 µm) for 15, 30, 60 or 90 min before fixation using flow cytometry. Anti-CD42b-DyLight 649 was added together with SYTO13 before fixation (A, $n = 3$) or after SYTO 13 incubation and fixation (B, $n = 4$). Representative plots are shown for 15 and 90 min each. Results are presented as mean ± SD. Statistical analysis was performed using repeated measures one-way analysis of variance with Bonferroni multiple comparison test (*versus* 15 min). ns, not significant; RP, reticulated platelets.

lysing red blood cells (RBCs) and fixing cells. This yielded significantly improved fluorescence intensity, enhancing staining quality and separation (Figure 2B,D), without affecting CD42b expression (Figure S1B). Based on these results, we chose a final concentration of 25 µM SYTO 13, added post-RBCs lysis and cell fixation.

Circulating RP levels in a steady-state and under conditions of increased RP production

To evaluate the efficacy of SYTO 13 for RP identification, we compared circulating RP levels at baseline and in platelet-depleted mice during the recovery phase (D4 after anti-CD42b treatment). Successful platelet depletion and recovery was confirmed on D1 (platelet count: $43 \pm 57 \times 1000/\mu L$, $n = 9$) and D4 ($485 \pm 204 \times 1000/\mu L$, $n = 9$, $p = .0003$), respectively (Figure S2). In untreated mice, the average baseline level of circulating RP was $11\% \pm 1\%$ and significantly increased to $35\% \pm 13\%$ on D4 post platelet depletion ($n = 9$, $p < .0001$, Figure 3).

Characterization of murine RP

Building on our RP detection method, we explored the surface expression of the platelet activation marker P-selectin in murine RP and non-RP using untreated mice.

In unstimulated samples, P-selectin expression was < 1% for both RP and non-RP ($n = 6$, Figure 4), indicating no pre-activation. Upon stimulation with 400 µM PAR-4 agonist, the percentage of P-selectin positive platelets increased significantly higher in RP ($93\% \pm 6\%$) compared to non-RP ($77\% \pm 14\%$, $n = 6$, $p = .0065$, Figure 4). Additionally, RP showed significantly higher geometric mean fluorescence intensity levels *versus* non-RP (1874 ± 1278 *versus* 859 ± 549, $n = 6$, $p = .02$, Figure 4).

Discussion

In this study, we assessed the effectiveness of the nucleic acid dye SYTO 13 in detecting murine RP in diluted blood, examining both, steady-state and a condition with heightened RP production.

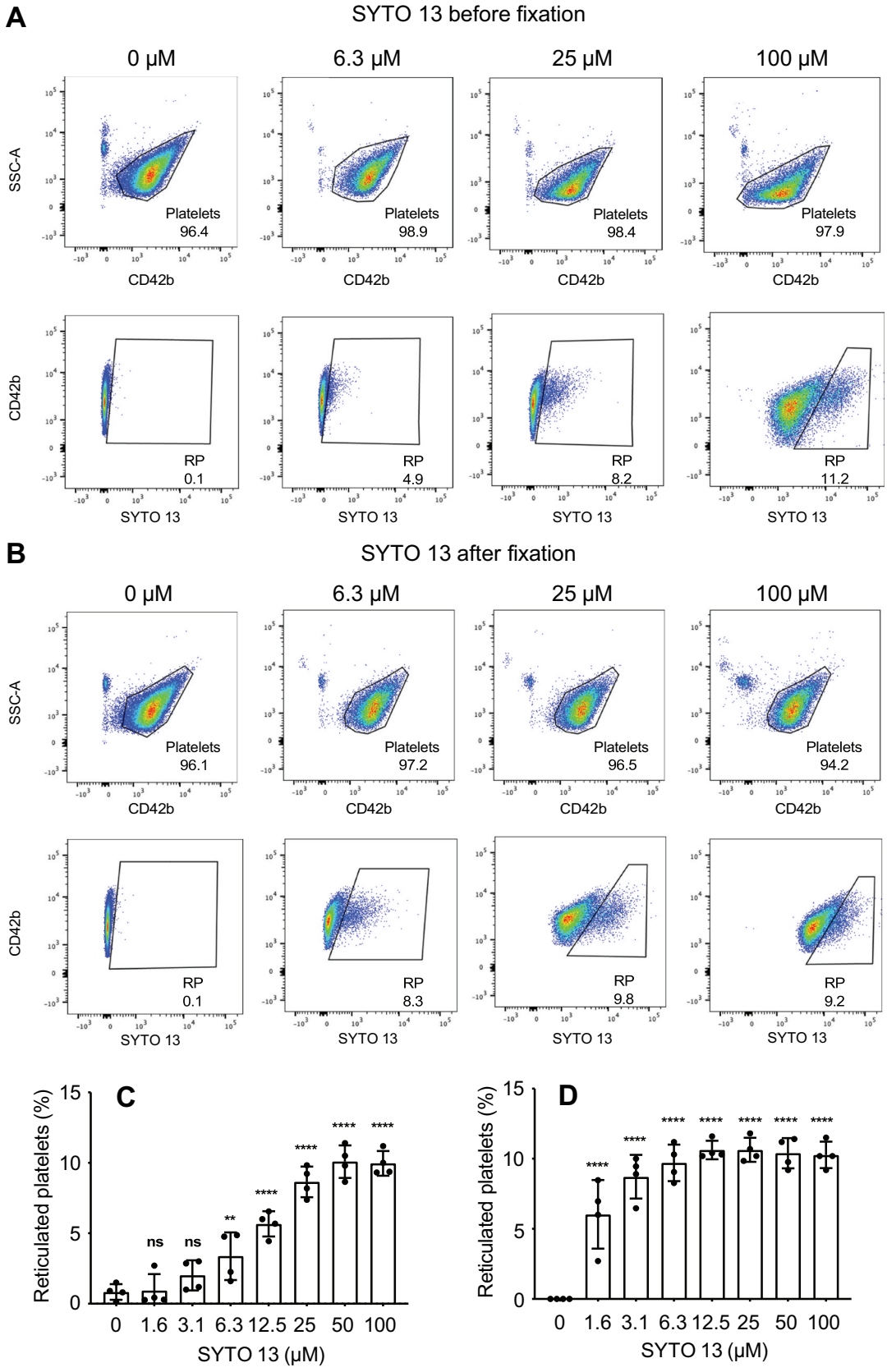

Figure 2. Analysis of reticulated platelets with SYTO 13 titration before or after fixation. The percentage of reticulated platelets was determined at baseline in anticoagulated blood (1:10 diluted) following incubation with PBS (0 µm) or different SYTO 13 concentrations (ranging from 1.6–100 µm) for 15 min before (A, C, $n = 4$) or after (B, D, $n = 4$) fixation using flow cytometry. Anti-CD42b-DyLight 649 was added after fixation. Representative plots are shown for specific SYTO 13 concentrations (0, 6.3, 25 and 100 µm). Results are presented as mean ± SD. Statistical analysis was performed using repeated measures one-way analysis of variance with Bonferroni multiple comparison test (*versus* PBS). $^{**}p < .01$; $^{****}p < .0001$; ns, not significant; RP, reticulated platelets.

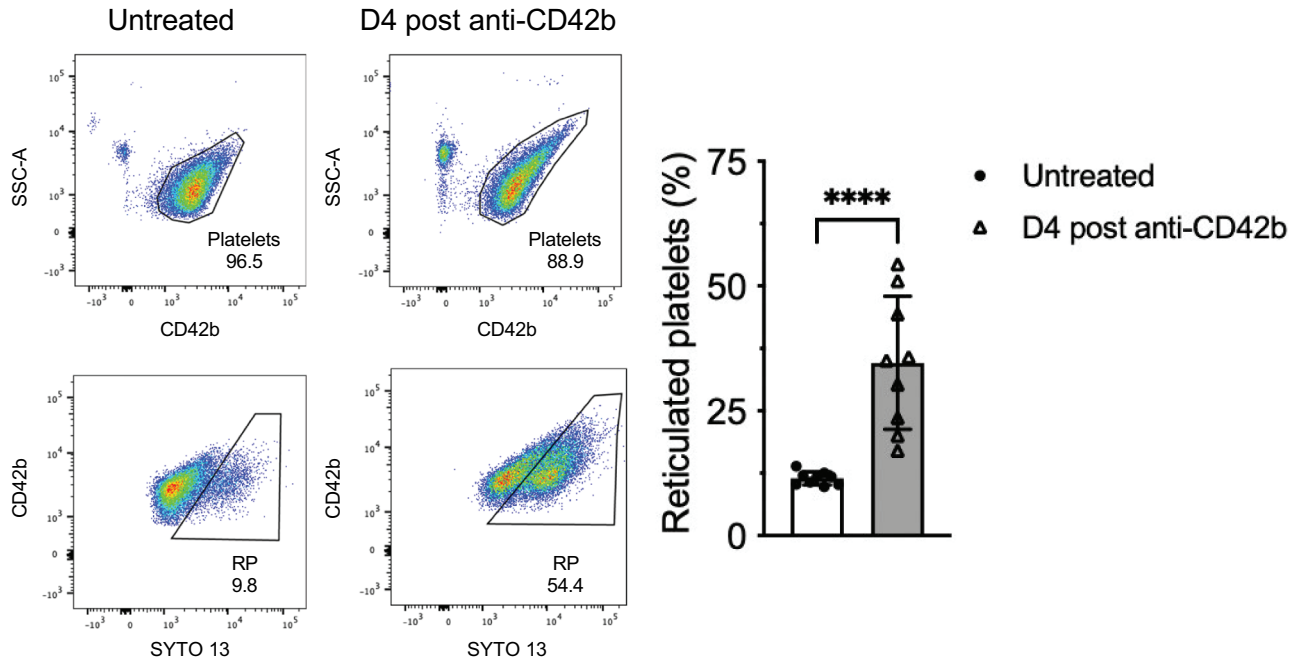

Figure 3. Analysis of reticulated platelets in mice treated with anti-CD42b compared to untreated mice. The percentage of reticulated platelets was determined in anticoagulated blood (1:10 diluted) using SYTO 13 (25 µm, 15 min) and the platelet labeling antibody anti-CD42b-DyLight 649 in untreated mice ($n = 9$), or mice treated with the platelet-depletion antibody R300 (anti-CD42b, Emfret) during recovery (day 4 after treatment) ($n = 9$) using flow cytometry. Representative plots are shown for an untreated mouse and a mouse on day 4 post-treatment. Results are presented as mean ± SD. Statistical analysis was performed using unpaired t-test. ****$p < .0001$; D4, day 4; RP, reticulated platelets.

Our flow-cytometric method enables a clear distinguishment between RP and non-RP and the parallel analysis of platelet surface markers.

In untreated mice, the percentage of platelets with a high SYTO 13 signal (about 10%, Figure 2B and Figure 3) corresponds to the expected values for RP known from the literature.[4] Notably, RP (SYTO-high platelets) separated well from non-RP (SYTO-low platelets) in flow cytometry gating, without the necessity to set an arbitrary gate for RNA-high platelets as done in previous human and murine RP staining protocols.[7,9,11] The optimal SYTO 13 concentration (25 µM), allowing for determining a distinct RP population, was achieved by SYTO 13 titration and incubating SYTO 13 after platelet fixation (Figure 2). The relatively high concentration of SYTO 13 was required most likely due to the presence of leukocytes in the whole blood assay. Leukocytes contain a much higher RNA (and DNA) content compared to platelets, thus competing with SYTO 13 uptake. SYTO 13 dyes are supposed to be nontoxic and cell-permeable, and thus suitable for staining living cells.[13] However, when using 25 µM SYTO 13 without prior fixation, we observed a reduction in the surface expression of the specific platelet marker GPIbα (CD42b) (Figure S1A). Therefore, we recommend applying higher SYTO 13 concentrations only after platelet fixation to exclude unwanted effects. In experiments where fixation is unwanted, e.g., RP sorting and downstream analyses, it might still be possible to use lower amounts of SYTO 13 without affecting platelets, especially when staining washed platelets in the absence of leukocytes.

As we combine SYTO13 with a specific platelet marker, the RP analysis can be performed in whole blood without prior platelet isolation, preventing potential platelet activation during the extensive washing procedure. Moreover, the use of whole blood allows the parallel assessment of leukocytes if desired. Our protocol is fast, including 15 min of lysis/fixation, 5 min of washing, 15 min of staining with SYTO 13 and anti-CD42b-DyLight 649, and a final wash of 5 min. An incubation time of 15 min with SYTO 13 was sufficient for effective staining. However, the detected RP values remained stable over a duration of 90 min. Therefore, the incubation time could be expanded, for example if SYTO 13 needed to be used under non-fixing conditions (Figure 1B). It is possible to add further markers to our protocol before or after fixation, depending on the fluorescent antibody. While there was no interference with anti-CD62P-PE/Cy7 when added before fixation, we observed a potential platelet aggregate formation with anti-CD42b-DyLight 649 (Figure 1A). Therefore, we recommend applying the latter antibody only after fixation.

SYTO 13 is not a specific RNA dye; it also stains DNA.[8] While platelets are anucleate, they contain mitochondrial DNA,[14] and might have residual genomic DNA originating from megakaryocytes.[15] To validate the specificity of SYTO 13 for RP detection, we employed a murine platelet depletion method by injecting antibodies directed against GPIbα. This approach led to a notable decline in platelet counts followed by recovery (Figure S2), with a substantial increase in the fraction of platelets with a high SYTO 13 signal, i.e., RP (Figure 3). This is in agreement with previous findings,[11] and the assumption that RP are the youngest platelet fraction that has just been released from the bone marrow.[16,17]

Furthermore, human studies report that RP are associated with increased reactivity,[3,18–20] and RP exhibited higher P-selectin surface expression following stimulation with thrombin receptor activating peptide (TRAP) or adenosine diphosphate (ADP) *versus* non-RP.[21] Accordingly, in our mouse study, we observed a notable increase in P-selectin surface expression levels in RP compared to non-RP upon *in vitro* stimulation with the thrombin receptor (PAR-4) agonist (Figure 4). Similarly, other platelet activation markers or extra-/intracellular targets of interest could be analyzed in SYTO-labeled RP to characterize RP in different mouse models. Moreover, SYTO dyes are available in several

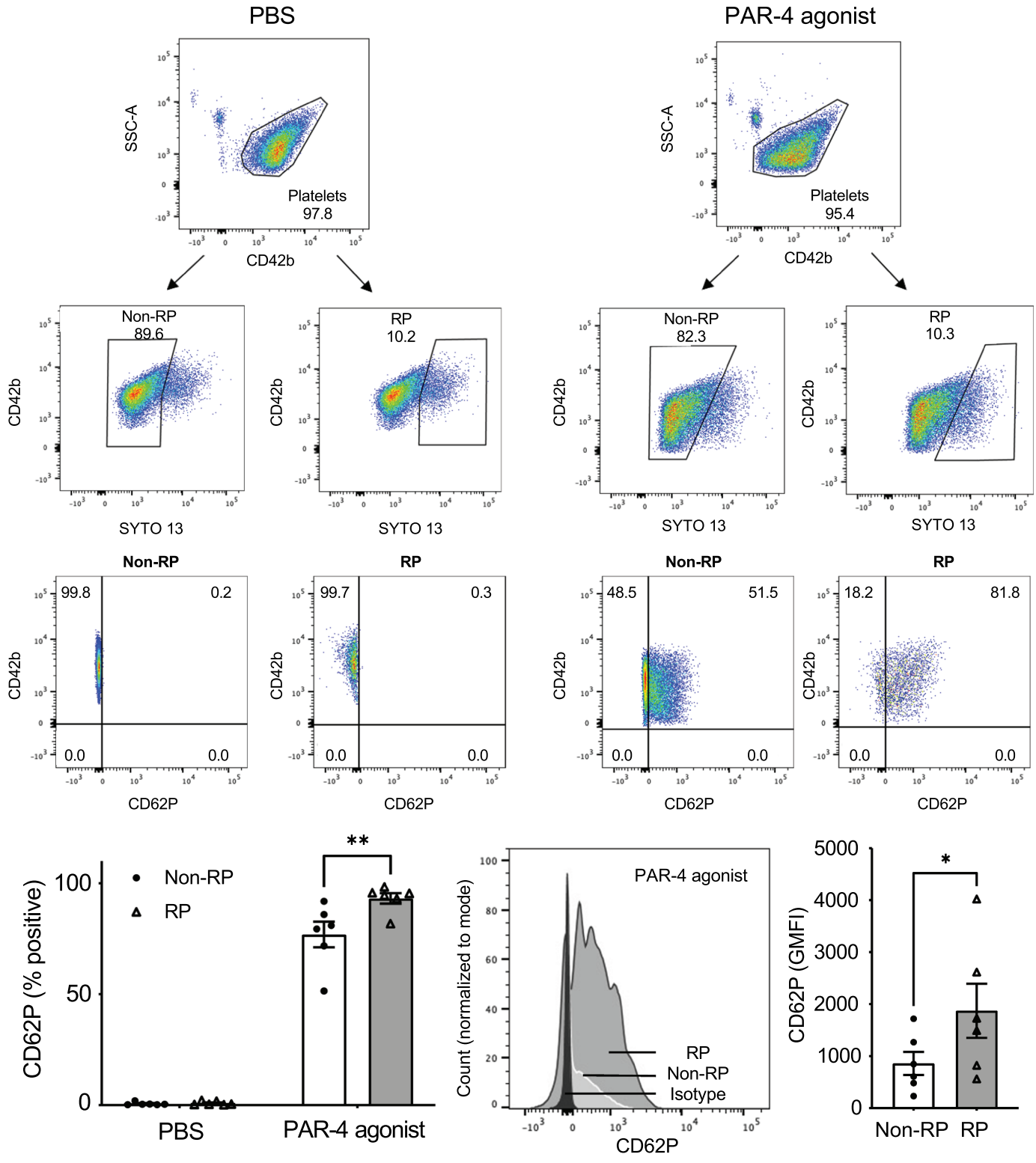

Figure 4. Analysis of the surface activation marker P-selectin (CD62P) in platelets stained with SYTO 13, in both their resting and stimulated states. The surface expression of the alpha granule component P-selectin of murine RP and non-RP was determined in anticoagulated blood (1:10 diluted) in untreated mice in the resting state (PBS, $n = 6$), or following stimulation with PAR-4 agonist (400 µm, 15 min, $n = 6$) using flow cytometry. Non-RP and RP were gated based on staining with SYTO 13 (25 µm, 15 min) and anti-CD42b-DyLight 649. Representative plots are shown for PBS and PAR-4 agonist treated samples and a representative histogram is shown for PAR-4 agonist treatment. Results are presented as mean ± SD. Statistical analysis was performed using paired t-test. *$p < .05$; **$p < .01$; GMFI, geometric mean fluorescence intensity; PAR-4, protease-activated receptor-4; PBS, phosphate-buffered saline; RP, reticulated platelets.

colors, which enables flexible combination with various fluorescently-labeled antibodies.

Whereas SYTO 13 is beneficial for *in vitro* RP detection by flow cytometry, it might not be useful for *in vivo* imaging or RP staining in tissue sections as the SYTO 13 signal of other cells with a prominent nucleic acid content would cover up the relatively small SYTO 13 signal originating from RP. To address this, genetically engineered mouse models with RP-specific labeling options are necessary to more

precisely identify RP and minimize the background staining of nucleated cells and non-RP. Furthermore, transmission electron microscopy could be employed to assess RP features.

Conclusion

Here, we provide a method for *in vitro* detection of murine RP in diluted blood using SYTO 13 and flow cytometry. The protocol is short and adjustable. SYTO 13 is commercially available offering stable and reproducible RP values with the possibility to stain for other markers simultaneously. Our findings affirm SYTO 13's reliability, aligning with the literature on RP increase following platelet depletion and RP hyperreactivity. In conclusion, our study underscores SYTO 13 as a powerful tool for studying the role of RP in health and disease using murine models.

Disclosure statement

The authors report there are no competing interests to declare. Thomas G. Nührenberg is an employee of Novartis Pharma AG, Basel, Switzerland.

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Supplementary material

Supplemental data for this article can be accessed online at https://doi.org/10.1080/09537104.2025.2489020

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