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Evaluation of an Alternative Staining Method Using SYTO 13 to Determine Reticulated Platelets

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Abstract

Reticulated platelets reflect the rate of platelet turnover and represent the youngest circulating platelets in peripheral blood. Reticulated platelets contain residual ribonucleic acid (RNA) from megakaryocytes which is lost in a time-dependent manner and can be transcribed into proteins even in the absence of a nucleus. An increased proportion of reticulated platelets is associated with higher platelet reactivity, cardiovascular events and mortality. At present, a fully automated assay system (SYSMEX haematology analyser) is available for analysis. This method, however, is not suitable for extended laboratory investigations like subsequent cell sorting. Flow cytometry analysis after staining with thiazole orange (TO) is frequently used in such settings despite several limitations. Here, we describe a new assay for determination of reticulated platelets by flow cytometry using the nucleic acid staining dye SYTO 13 and compare it with SYSMEX and TO staining as current standards. A significant correlation between immature platelet fraction (IPF) determined by SYSMEX XE-2100 analyser and results obtained with the SYTO 13-based assay was observed ($r = 0.668$, $p < 0.001$) which was stable during a reasonable time period. In contrast, the correlation between TO staining and IPF was weaker ($r = 0.478$, $p = 0.029$) and lost after 90 minutes of staining. SYTO 13 staining of platelets enabled sorting of RNAlow and RNArich platelets which was confirmed by RNA quantification of sorted platelets. Except for fixation of platelets, sorting of these platelet sub-populations was stable under various experimental settings. In summary, determination of reticulated platelets with the new SYTO 13 assay offers distinct technical advantages enabling further laboratory processing.

Keywords
- reticulated platelets
- immature platelet fraction
- staining

Introduction

Reticulated platelets (RPs), also known as immature platelets, are a fraction of the platelet pool, mostly representing the youngest platelets released from the bone marrow.[1,2] They are characterised by some—known so far—distinct features such as: increased ribonucleic acid (RNA) content, higher volume, more dense granules, higher levels of surface activation markers and probably higher platelet reactivity.[1,3–6] Until now, several studies have shown an association of RP levels and cardiovascular events or mortality.[7–10] Several laboratory methods with distinct features are used to determine RP in clinical settings. Initially, analysis of RP was performed by means of light microscopy using supravital staining of blood with new methylene blue, but this method is obviously not well suited for extended applications.[11] Currently, flow cytometry is the method preferred by the majority of clinical laboratories.

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Kienast and Schmitz reported a flow cytometric assay based on thiazole orange (TO) staining for analysing RP.[12] TO binds to platelet RNA and various intracellular components.[13] The amount of unspecific non-RNA binding and hence varying fluorescence intensity is affected by staining conditions, such as incubation time, temperature or fixation.[1] Consequently, this method is hampered by an evident lack of analytical standardization, which disables a direct comparison of data obtained by different laboratories.[4,12,14–16] Moreover, when prolonged sample work-up is inevitable, for example, in the setting of cell sorting with a low number of events, TO staining is difficult as it leads to unspecific TO-positive platelets and increasing fluorescence intensity over time.

An alternative option to measure RP is a fully automated assay using a fluorescent polymethine dye which has been established on the SYSMEX XE-2100 (SYSMEX, Kobe, Japan) analyser. The immature platelet fraction (IPF, %) provides the fraction of immature platelets within the whole platelet pool, while immature platelet count provides the number of immature platelets (10^3/µL)—both determined using a cut-off pre-defined by the manufacturer. By means of this method, an association between RP and clinical events or drug response, for example, in patients treated with thienopyridines, has previously been shown.[5,8–10,17] However, as the SYSMEX assay is a fully automated closed system, assay modifications for experimental purposes can hardly be implemented and more in-depth investigations on RP surface markers are not possible.

Therefore, the aim of our present work is to establish a new platelet staining protocol which overcomes some of the limitations in RP analysis mentioned before. A potential staining dye is SYTO 13 which is a cell-permeant green fluorescent dye with high potency and affinity to RNA showing a large fluorescence enhancement after binding. SYTO 13 has an absorption maximum at 491 nm and an emission maximum at 514 nm in the presence of RNA.

Materials and Methods

Staining of Platelets

We developed a laboratory protocol for SYTO 13 staining of platelets. First, washed platelets (WPs) were obtained from citrate-anticoagulated blood (Sarstedt, Nümbrecht, Germany). Blood was centrifuged at 150 × *g* for 10 minutes and platelet-rich plasma (PRP) was manually separated. To avoid activation and aggregation of platelets, 10 µL prostaglandin I_2 (10 µg/mL; Sigma-Aldrich, St. Louis, Missouri, United States) were added to 490 µL PRP and thoroughly mixed. After pelleting (380 × g, 20 minutes), platelets were re-suspended in 1,000 µL Tyrode's-HEPES buffer (pH 7.4; 145 mMol NaCl [Sigma-Aldrich], 2.9 mMol KCl [Sigma-Aldrich], 10 mMol HEPES [Sigma-Aldrich], 1 mMol $MgCl_2$ [Sigma-Aldrich], 5 mMol glucose [Sigma-Aldrich]), pelleted again (380 g, 20 minutes) and re-suspended in 500 µL Tyrode's-HEPES buffer. Platelets were diluted to a final concentration of 5 × 10^4 platelet/µL with Tyrode's-HEPES buffer. To stain RNA, 10 µL of SYTO 13 (1 µM, final concentration 12.5 nM; Thermo Fisher, Waltham, Massachusetts, United States) were added to a suspension of 100 µL WP diluted in 690 µL phosphate-buffered saline (PBS; pH 7.4; 137 mMol NaCl, 2.7 mMol KCl, 10 mMol Na_2HPO_4 [Sigma-Aldrich], 1.8 mMol KH_2PO_4 [EMD Millipore, Burlington, Massachusetts, United States]). For TO staining, 70 µL of Retic-Count (Becton Dickinson, Heidelberg, Germany) was added to a suspension of 100 µL WP diluted in 630 µL PBS. As negative controls, 100 µL WP were diluted in 700 µL PBS. After respective periods of incubation at room temperature in the dark, median fluorescence intensity (MFI, arbitrary units) of all platelets was measured using a SONY SH800Z cell sorter (SONY, Tokyo, Japan).

Furthermore, modifiability of assays is a desirable criterion for laboratory experiments. Hence, we compared staining of WP (a), fixed WP (b), PRP (c) and whole blood (d) to confirm adaptability of SYTO 13 staining of platelets:

(a) WP were prepared and stained for 90 minutes as aforementioned.
(b) After fixation of WP with 1% formaldehyde (Polysciences, Hirschberg, Germany) for 10 minutes, 100 µL fixed WPs were stained with 10 µL SYTO 13 (1 µM) in 690 µL PBS for 90 minutes.
(c) 50 µL PRP were stained with 10 µL SYTO 13 (1 µM) in 690 µL PBS for 90 minutes.
(d) 5 µL whole blood were double-stained with 5 µL CD41 allophycocyanin (APC) (Becton Dickinson) as a platelet-specific marker and 10 µL SYTO 13 (1 µM) in 690 µL PBS for 90 minutes.

All steps were performed at room temperature unless indicated otherwise. Fluorescence was quantified in the FL-1 fluorescein isothiocyanate channel (500–550 nm) after platelet gating by forward scatter/backward scatter characteristics and doublet exclusion. For whole blood staining, platelets were additionally identified by gating for CD41-positivity in the FL-4 APC (650–680 nm) channel. Fluorescence intensities of unstained platelets remained stable over at least 24 hours. The sorter was calibrated with automatic setup beads (SONY) every day before use.

Platelet RNA Stability at Different Incubation Temperatures

RNA of unstained WP from three healthy donors (19.5 × 10^6, 21 × 10^6, 23 × 10^6 platelets) was isolated immediately after WP preparation as well as after 5 hours of storage at 22°C or 37°C, respectively. 500 µL WP were diluted 1:4 with TRIzol LS reagent (Thermo Fisher). 200 µL chloroform (Carl Roth, Karlsruhe, Germany) per 750 µL TRIzol LS were added. After 3 minutes of incubation, samples were centrifuged for 15 minutes at 12,000 × *g* at 4°C. The aqueous phase was manually separated. Thereafter, RNA isolation from the aqueous phase was accomplished by use of a miRNeasy Micro Kit (Qiagen, Venlo, Netherlands) per protocol of the manufacturer. The extracted RNA amount was determined by means of a RNA 6000 Pico Assay on a 2100 Bioanalyzer (Agilent, Santa Clara, California, United States).

Correlation of TO/SYTO 13 Staining to IPF

Blood samples obtained from 21 patients after transcatheter aortic valve implantation (TAVI) were analysed. Their

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median age was 83 years (interquartile range [IQR]: 80–86). Eighteen patients received Edwards-Sapien 3 aortic valves and 3 patients received Evolute R aortic valves, respectively. Demographic and clinical data of these patients are provided in ►**Supplementary Table S1** (available in the online version). The study was approved by the ethics committee of the University of Freiburg (Germany) and all patients gave written informed consent before any study procedure. Blood was collected 1 day following the TAVI procedure and WP were stained with TO and SYTO 13 as described afore. Polymethine-based RP analysis as well as IPF were determined using the automated blood cell counter SYSMEX XE-2100. Spearman's ρ was used to assess correlations between IPF and MFI of TO- and SYTO 13-stained platelets.

RNA Quantification according to SYTO 13 Staining Intensities

For internal validation, the association of RNA content and SYTO 13 staining intensities was investigated. SYTO 13-stained platelets obtained from a drug-free healthy subject were divided into quintiles in the FL-1 channel according to increasing fluorescence intensities. A total of 8×10^6 platelets were sorted from each gate and RNA was extracted as described before. Due to the high amount of fluid, the first centrifugation step in the Qiagen protocol was modified and a vacuum chamber was used instead for separation of RNA. The next steps were performed per protocol of the manufacturer. The extracted RNA amount was determined in duplicate as described before.

RNA Quantification of RNAlow and RNArich Platelets

As a second internal validation, the RNA amount of 8×10^6 platelets sorted from the outer quintiles of SYTO 13 fluorescence intensities (termed RNAlow and RNArich platelets) was quantified in WP from eight healthy donors. RNA was extracted and quantified as described before. Paired *t*-test (GraphPad Prism 7, San Diego, California, United States) was used to compare RNA amounts of RNAlow and RNArich platelets. All values are expressed as median with IQR unless otherwise indicated.

Results

Staining of Washed Platelets

SYTO 13 staining of platelets showed a continuous shift in the FL-1 channel with—compared with TO staining—a remarkable 10-fold increase in mean fluorescence intensity (►**Fig. 1**). Doublet gating was used to avoid staining events with false positive high fluorescence intensity (►**Supplementary Fig. S1**, available in the online version).

Staining under Various Conditions

Furthermore, staining of fixed WP, PRP and whole blood showed that SYTO 13 staining is feasible under various conditions. Each tested condition yields an increase of fluorescence in the FL-1 channel (►**Fig. 2**, left two columns). In sorting experiments, SYTO 13 staining of platelets enables adequate sorting as confirmed by re-analysis of flow cytometry plots for RNArich and RNAlow platelets in different FL-1 gates (►**Fig. 2**, right two columns). Platelet fixation resulted in loss of MFI as compared with the other conditions. Depending on the planned specific laboratory experiments, one might choose the most appropriate condition.

Stability of Staining Intensity

Having demonstrated that SYTO 13 staining, as compared with TO staining, is feasible after 90 minutes of staining, the stability of staining intensity over prolonged incubation times was investigated. After staining for 90 minutes, fluorescence intensity remained stable for at least 5 hours (►**Fig. 3A**). RNA amount remained stable up to at least 5 hours of incubation at room temperature (22°C), while a substantial amount of RNA was degraded after 5 hours of incubation at 37°C (►**Fig. 3B**).

Correlation of TO and SYTO 13 Staining with IPF

MFI of SYTO 13-stained platelets correlated well with the established IPF measurement by SYSMEX analysed 30 minutes ($r = 0.665$, $p = 0.001$) as well as 90 minutes ($r = 0.668$, $p < 0.001$) after staining, whereas a moderate correlation with MFI of TO-stained platelets was obtained solely after 30 minutes ($r = 0.478$, $p = 0.029$; ►**Fig. 4**).

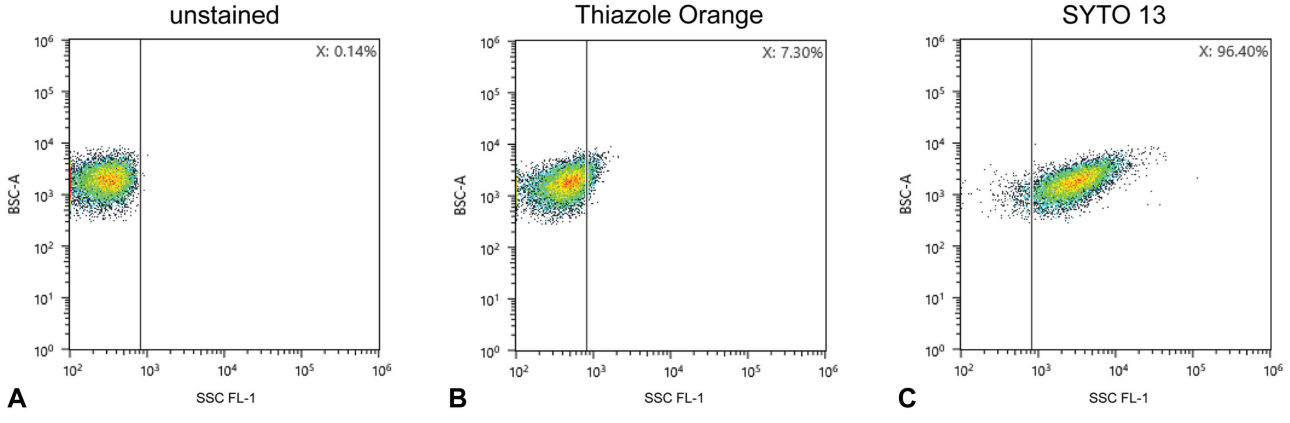

Fig. 1 Flow cytometry plots after an incubation time of 90 minutes of (**A**) unstained, (**B**) TO-stained and (C) SYTO 13-stained unfixed washed platelets from a patient after TAVI. Abbreviations: BSC, backward scatter; SSC, side scatter; FL-1, 500–550 nm; TAVI, transcatheter aortic valve implantation; TO, thiazole orange.

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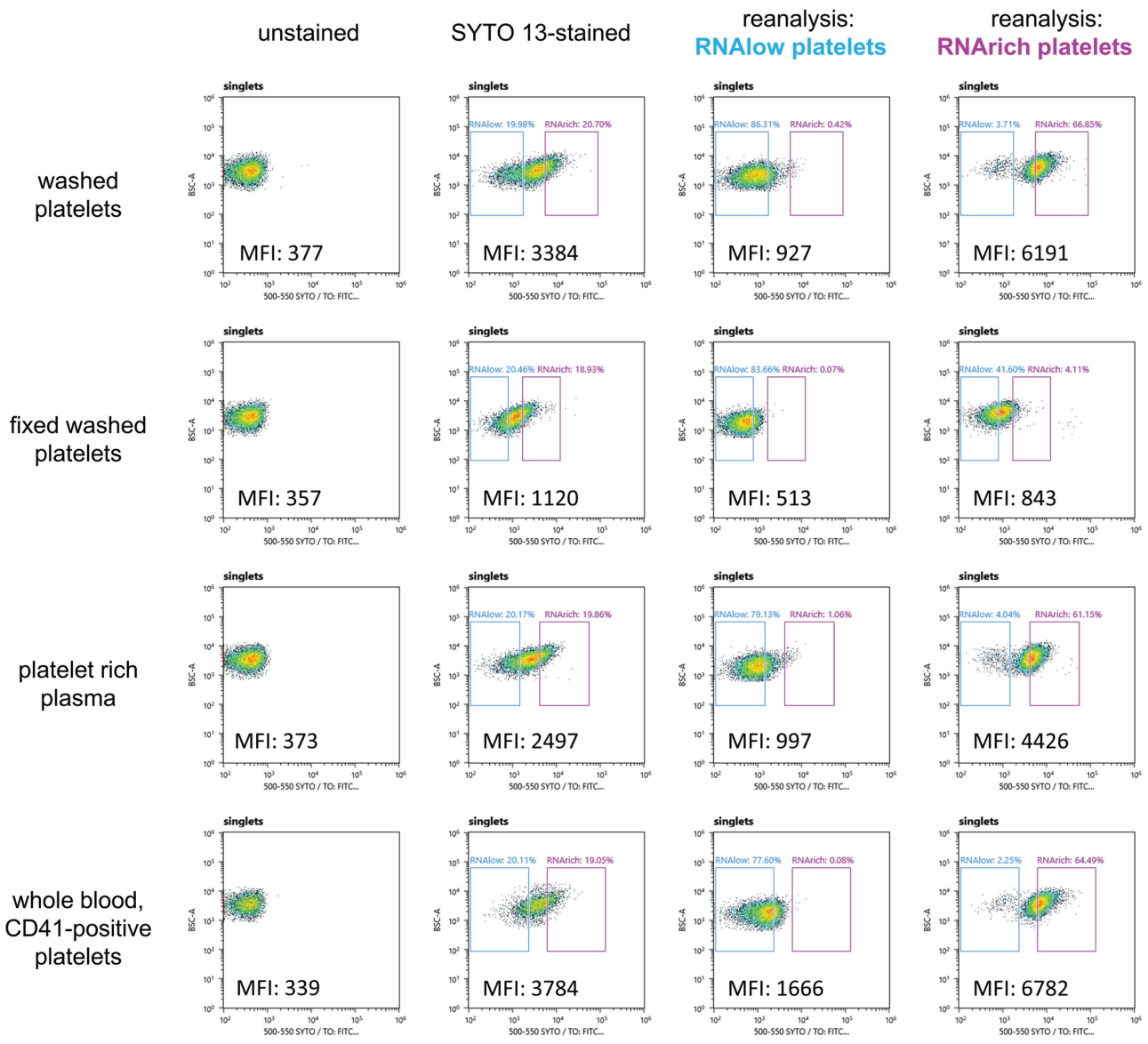

Fig. 2 Staining of washed platelets, fixed washed platelets, platelet-rich plasma and whole blood with SYTO 13 for 90 minutes. Platelets were sorted according to the RNAlow and RNArich gates and re-analysed. MFI, median fluorescence intensity (arbitrary units).

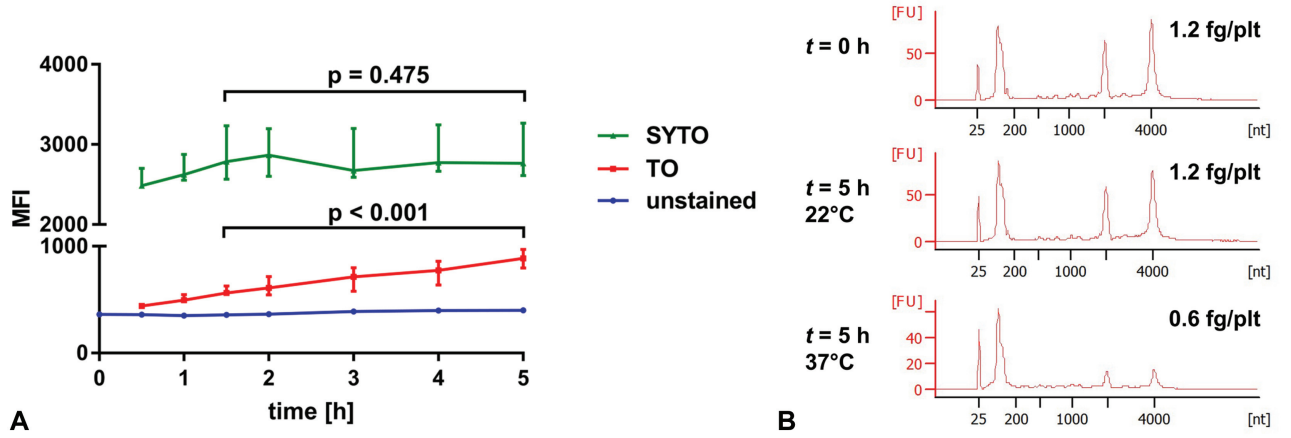

Fig. 3 (A) Stability of SYTO 13 and thiazole orange (TO) staining over time from 3 healthy subjects (median and range; h = hours). (B) Representative example of a Bioanalyzer electropherogram of washed platelet ribonucleic acid (RNA) from a healthy donor immediately after preparation of washed platelets and at 5 hours of storage at 22°C and 37°C, respectively. Abbreviation: MFI, median fluorescence intensity (arbitrary units).

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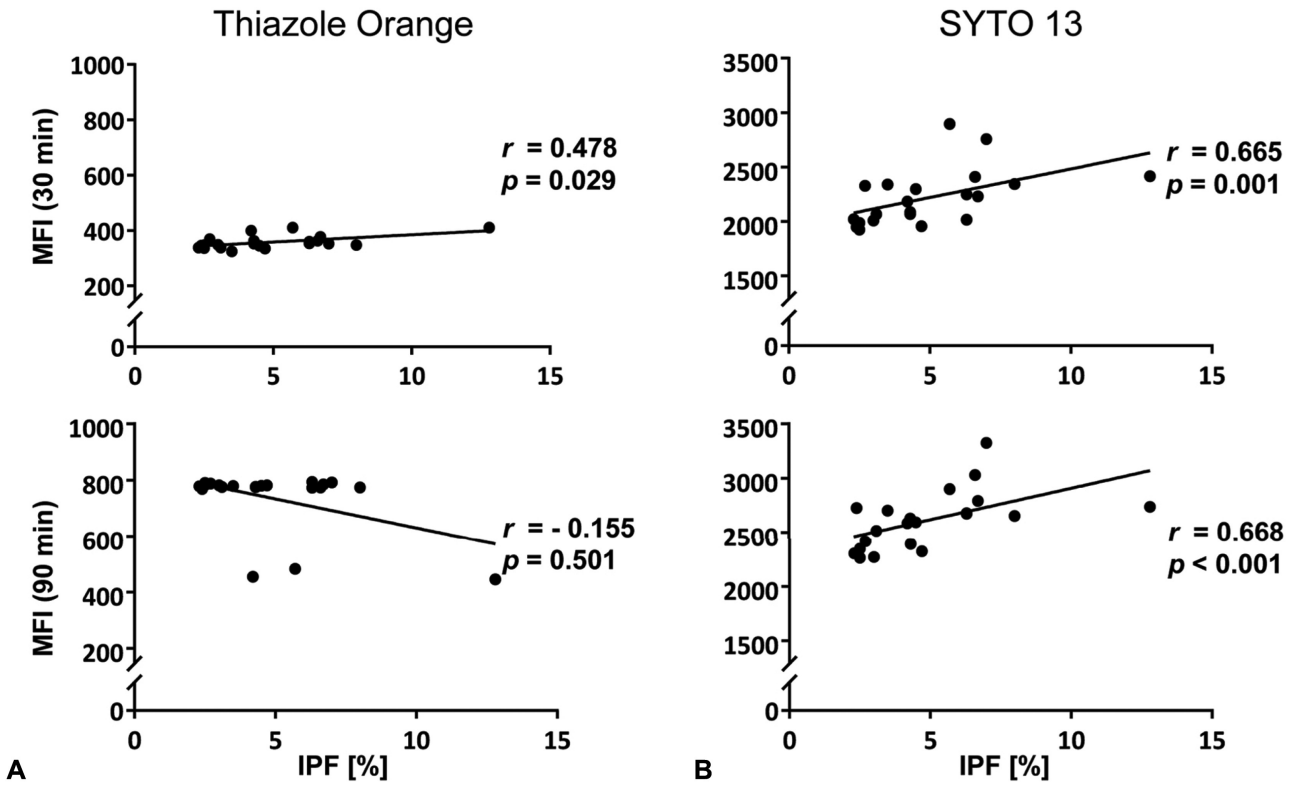

Fig. 4 Correlation of immature platelet fraction (IPF) and MFI of unfixed washed platelets after 30 minutes (upper panels) and 90 minutes (lower panels) of staining with (**A**) thiazole orange and (**B**) SYTO 13 ; $n = 21$ patients on day 1 after transcatheter aortic valve implantation. Abbreviation: MFI, median fluorescence intensity (arbitrary units).

RNA Quantification according to SYTO 13 Staining Intensities

RNA quantification of sorted platelets according to the quintiles of MFI showed a strong association of SYTO 13 staining intensities and extracted RNA amount (►**Fig. 5**). ►**Fig. 5B** shows Bioanalyzer electropherograms of sorted platelets from each quintile indicating an increase in the amount of total RNA with higher fluorescence intensity. 18S and 28S ribosomal peaks can only be observed in the electropherogram of the quintile with highest staining intensity which is indicative for the high RNA amount in this quintile.

RNA Quantification of RNAlow and RNArich Platelets

RNA quantification resulted in a RNA amount of 0.35 (0.23–0.52) fg per platelet in the 20% of platelets with lowest staining intensity (RNAlow platelets) and 0.88 (0.78–1.14) fg per platelet in the 20% of platelets with highest staining intensity (RNArich platelets; $p = 0.016$).

Discussion

Our approach was based on certain features: (1) the staining dye should be specific for a single component of the platelet, (2) the staining procedure should yield a clear increase in fluorescence intensity, (3) the staining conditions should enable additional staining of extra- and intracellular components with other dyes or antibodies, (4) advanced processing of platelets by cell sorting for further analysis should be feasible and (5) the staining should be comparable to the well standardized and clinically established method, the SYSMEX polymethine dye-based assay. We selected a nucleotide staining dye since this seems to fulfil all requirements outlined above.

RNA extraction and quantification of platelets divided into quintiles by fluorescence intensity after SYTO 13 staining showed a clear association of RNA amount and fluorescence intensity. Distinct ribosomal peaks could only be determined in the electropherogram of the quintile with highest fluorescence intensity indicating that RPs are present in this quintile. Hence, the quintile with highest fluorescence intensity may be adopted as a gate for dedicated sorting of RP. RNA extraction and quantification of sorted RNAlow and RNArich platelets confirm highly different amounts of RNA in the respective populations. Therefore, our staining and gating method indeed identifies different platelet populations having low and high RNA levels correctly. In contrast to TO staining, the stability of SYTO 13 MFI facilitates analysis of stained platelets in extended experimental settings, for example, during cell sorting with low event rates.

Despite the strong association of SYTO 13 fluorescence intensities and extracted RNA amount, we cannot exclude potential unspecific labelling of other cell components, for example, dense granules or mitochondria. From the current data, similar to other methods with continuous staining distribution, the true rate of newly released platelets per day remains undetermined. Detailed in vivo experiments, for example, radiolabelling, would be necessary to define specific cut-off values for platelet turnover. In summary, our results indicate that SYTO 13 seems to be an attractive alternative staining dye for experiments on RNA-rich platelets and thereby also for RP.

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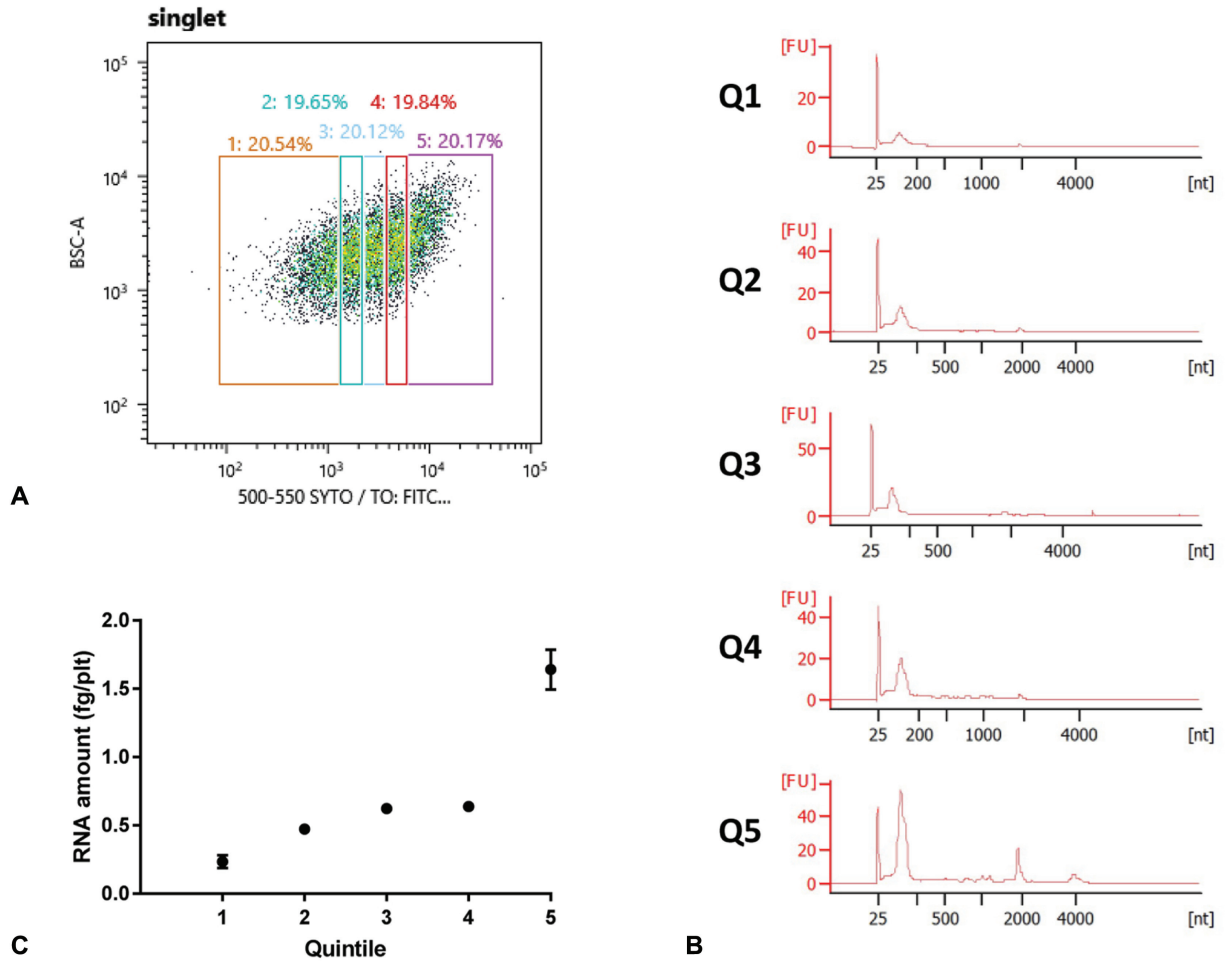

Fig. 5 (**A**) Gating strategy for sorting unfixed washed platelets in the FL-1 channel according to quintiles ranging from low to high fluorescence intensities after SYTO 13 staining (incubation time: 90 minutes). (**B**) Bioanalyzer electropherograms of extracted platelet ribonucleic acid (RNA) from each quintile. (**C**) RNA amount (fg per platelet) of sorted platelets from each quintile; platelets from a drug-free healthy subject measured in duplicate.

What is known about this topic?

- A higher immature platelet fraction (IPF) is linked to higher platelet reactivity.
- Reticulated platelets are associated with increased cardiovascular events and mortality.
- There is no generally accepted standardised protocol for the determination of reticulated platelets using thiazole orange (TO) despite this is the so far most frequently used non-automated laboratory method.

What does this paper add?

- An alternative and highly stable staining method for reticulated platelets using SYTO 13 is described.
- Fluorescence intensities of SYTO 13-stained platelets are associated with the clinically so far best standardised laboratory parameter IPF.
- In contrast to TO staining, SYTO 13 staining facilitates analysis of platelets in extended experimental settings.

Note

Part of the data was presented as a poster at the Congress of the European Society of Cardiology 2017 in Barcelona (Spain).

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Conflict of Interest

Miss Hille reports grants from PharmCompNet Baden-Württemberg: Kompetenznetzwerk Pharmakologie Baden-Württemberg, grants from University Heart Center Freiburg - Bad Krozingen, during the conduct of the study.

Dr. Trenk reports grants fromPharmCompNet Baden-Württemberg:Kompetenznetzwerk Pharmakologie Baden-Württemberg during the conduct of the study; personal fees from Amgen, AstraZeneca, Bayer, Berlin Chemie, Boehringer

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Ingelheim, Bristol-Myers Squibb, Daiichi Sankyo, Pfizer and Sanofi, outside the submitted work.

Dr. Cederqvist, Dr. Hromek, Dr. Stratz and Dr. Nührenberg report no conflicts of interest.

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