Differently cell doublet profiles: comparison of light scattering with fluorescence measurement techniques

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INTRODUCTION

Doublet discrimination ensures the counting of single cells in your analysis. Doublet discrimination is usually based on pulse analysis of light scatter parameters. Pulse area (A) and pulse width (W) information can be used to discriminate a pulse originated from a single cell from a pulse originated from to cells stuck together. Fluorescence signals can be also used to discriminate aggregates, and are essential to identifying cells in the G2/M phase from doublets in the G0/G1 phase in cell cycle/DNA applications. The most used method combines FSC-A vs. FSC-H, whereas other strategies combine FSC- H vs. FSC-W and SSC-H vs. SSC-W. However, when studying activated or proliferating cells, scatter discrimination can be difficult. In this study we have compared the use of light scattering with fluorescence measurement techniques for successful doublet discrimination for single cells.

MATERIALS AND METHODS

EDTA anticoagulated peripheral blood from n=24 healthy donors was used for doublet discrimination purposes. Blood (50µL) was prepared with traditional flow cytometry methods with lysis of red blood cells using ammonium chloride, and the CyLyse reagent (Sysmex). To overcome lysing artifacts, no-lyse no-wash methods were also used. Leukocytes were stained with 10µM Vybrant™ DyeCycle™ Violet Stain (DCV, Thermo Fisher) protected from light in a dedicated water bath at 37ºC. Samples were run in duplicate using Attune™ and Attune™ NxT acoustic focusing flow cytometers. DCV was excited at 405 nm and its emission was collected using the following filter combination: 413 LP, 495 DLP, and 440/50 BP. DCV fluorescence was displayed in logarithmic scale. DCV threshold levels were set empirically using a SSC vs. DCV-H dual parameter plot to eliminate from detection the large amounts of red blood cells that are found in unlysed whole blood. Acquisition was stopped when 20,000 events were collected in the leukocyte gate. Statistical methods used in this study were predominantly comparative analysis (ANOVA and t-test for regression slope). Red cell NH_4Cl lysing treatment was performed for 10 min at RT. CyLyse (PFH-based) was used as defined by the manufacturer. Final volume of all tubes was adjusted to 2650µL using HBSS for flow cytometry acquisition.

RESULTS AND DISCUSSION I

We have compared different doublet discrimination methods under different experimental conditions. Pulse analysis of light scatter and fluorescence was compared using lyse-wash, lyse-no wash, and no-lyse no-wash methods. Representative plots for one of the comparisons are shown in Figure 1.
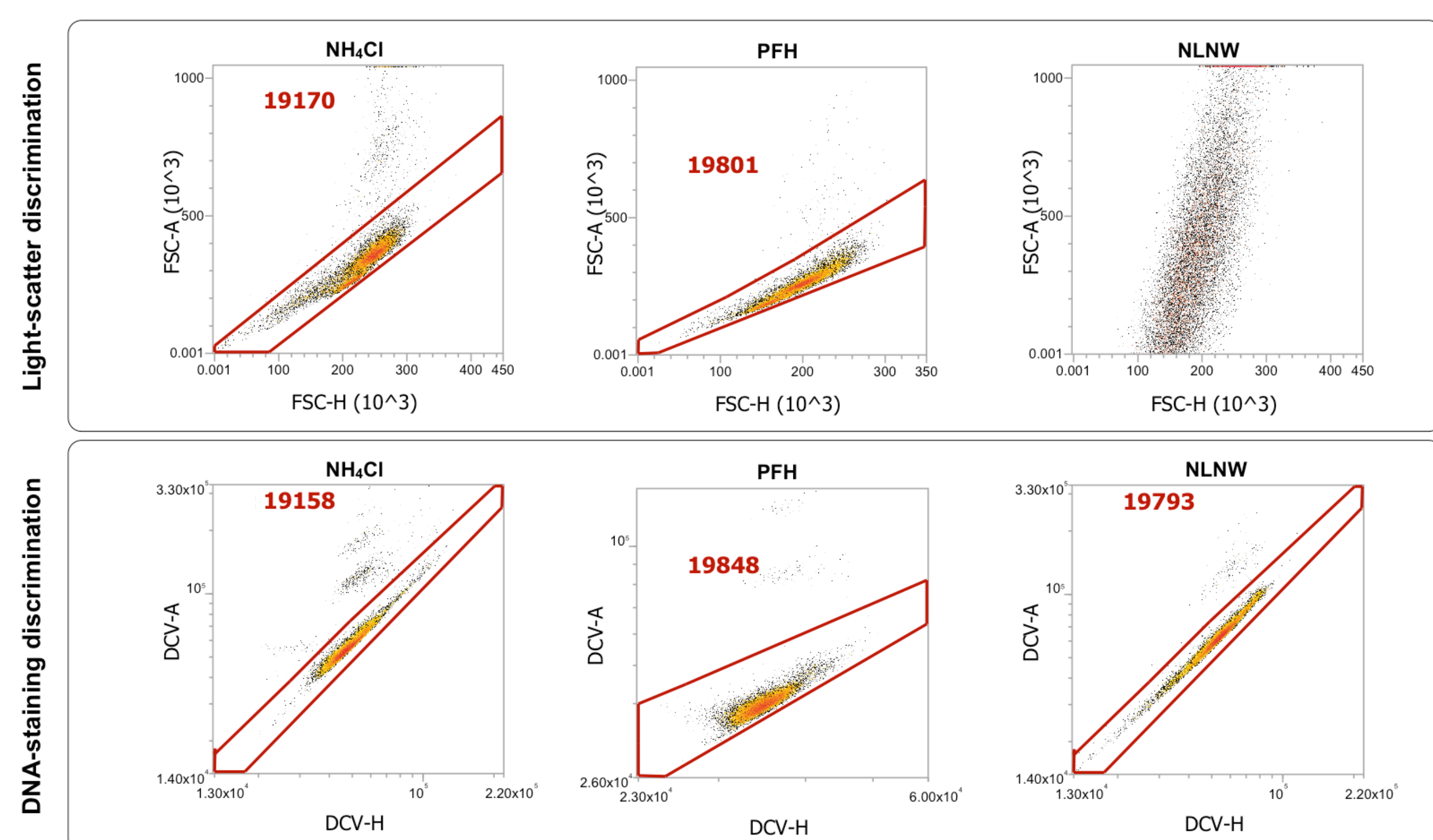

Figure 1. Region strategy to analyze the presence of aggregates by light scatter and fluorescence. Doublet discrimination comparisons based on light scatter parameters and DNA-fluorescent staining method under conditions: lyse-wash (NH_4Cl), lyse-no wash (PFH) and no-lyse no-wash (NLNW) methodologies. The statistics in the region represents count of the gate.

RESULTS AND DISCUSSION II

Single-cell screening of large quantity of cells can be difficult when pathological specimens are used. Doublet discrimination using DNA fluorescent dyes shows advantages over the light scatter based methods, especially for rare cell detection as well as for minimal residual disease studies. We also compared here the potential of viable DNA fluorescent dyes for doublet discrimination using leukapheresis products, showing that aggregates can be highly abundant and discriminated easily.
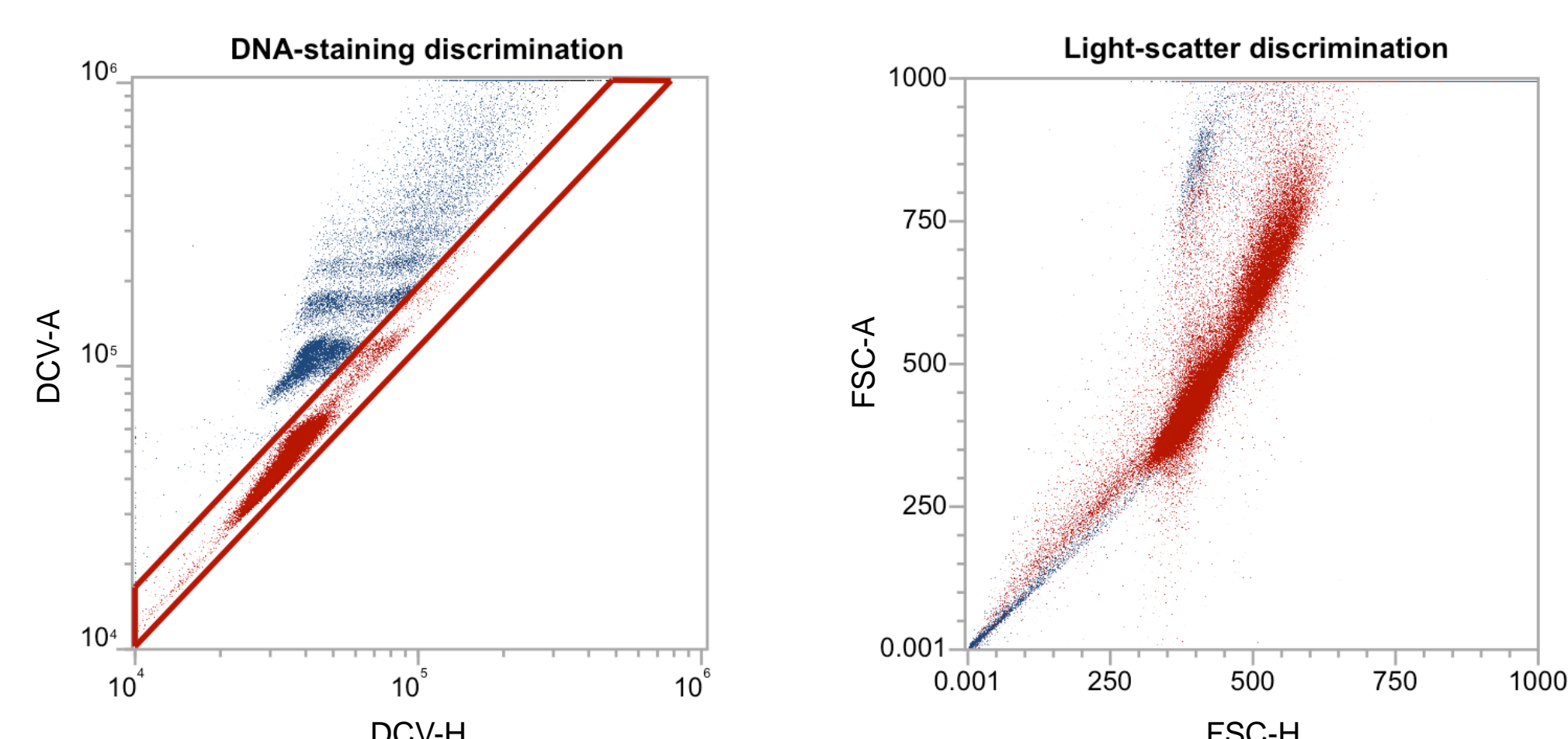

Figure 2. Comparison of light scattering with fluorescence measurement techniques for single-cell discrimination. Doublet discrimination using DNA viable fluorescent probes (i.e. DCV) is shown on the left and using light scatter parameters is shown on the right for a leukapheresis product.

RESULTS AND DISCUSSION III

Bland–Altman plots for method comparisons used 24 healthy hematopoietic samples. For each sample, the average cell concentration (cells/µL) was represented against the difference between cell counts obtained with the compared methods. The average ± 1.96 standard deviation of the difference (s.d.d.) value describes the 95% confidence interval for the difference between two methods. Comparisons between non-fixative (NH_4Cl) and PFH-based fixative solutions for red cell lysing, with no-lyse no-wash methods were also performed.
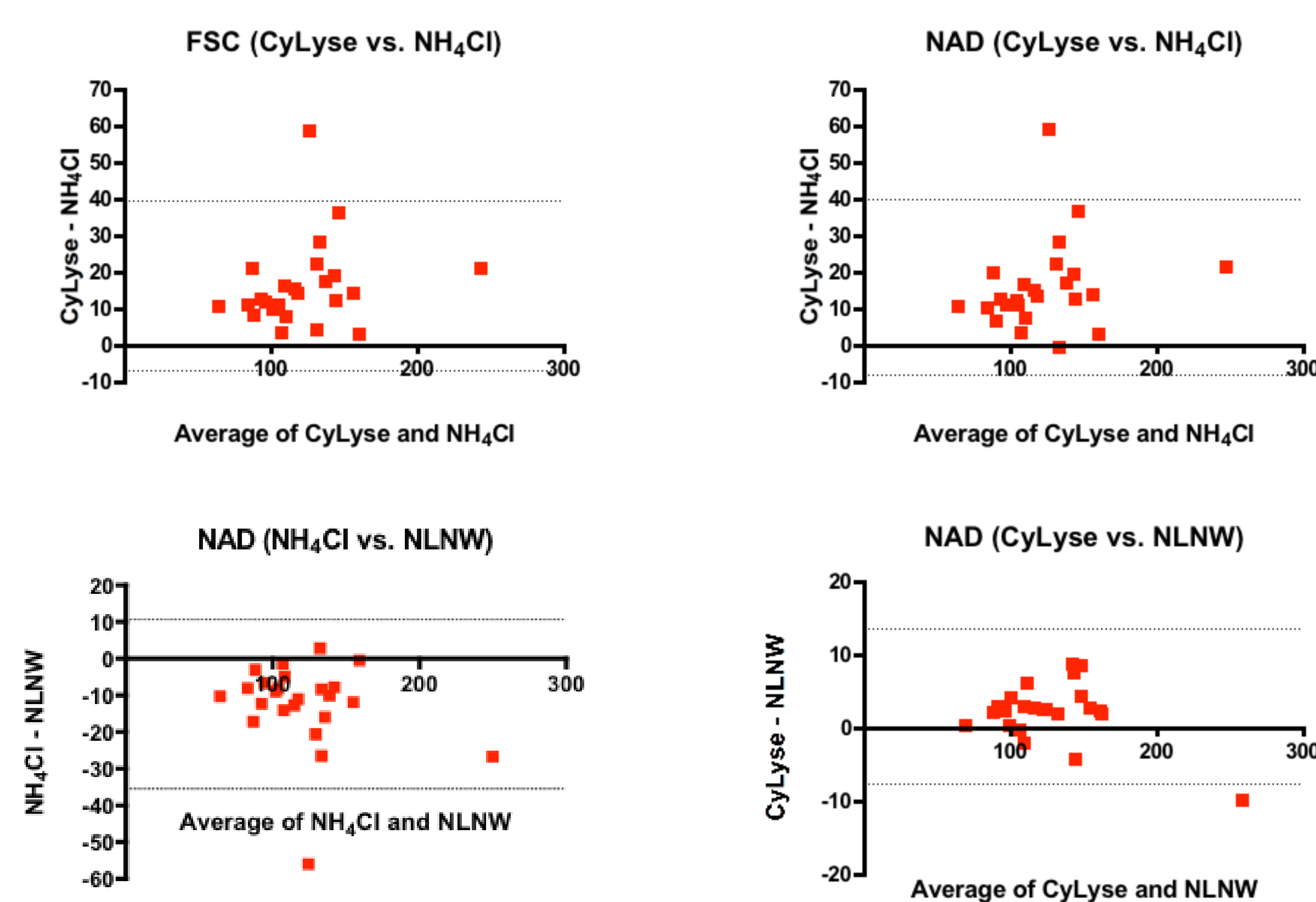

Figure 3. Bland–Altman plots for method comparisons using healthy hematopoietic samples. Bland-Altman comparison of ligh scatter (FSC) vs. Fluorescence (Nucleic Acid Dye, NAD) using NH_4Cl- and PFH-based red cell lysing solutions, and no-lyse no-wash methods (NLNW).

RESULTS AND DISCUSSION IV

Although lyse-no wash and no-lyse no-wash methods showed no significant differences in terms of cell concentration (One-way ANOVA), pairwise comparisons showed that the slope was near one (IC 95%, 0.89-0.93, P value = 0.0001) indicating that erythrocyte lysing solutions results in leukocyte depletion.
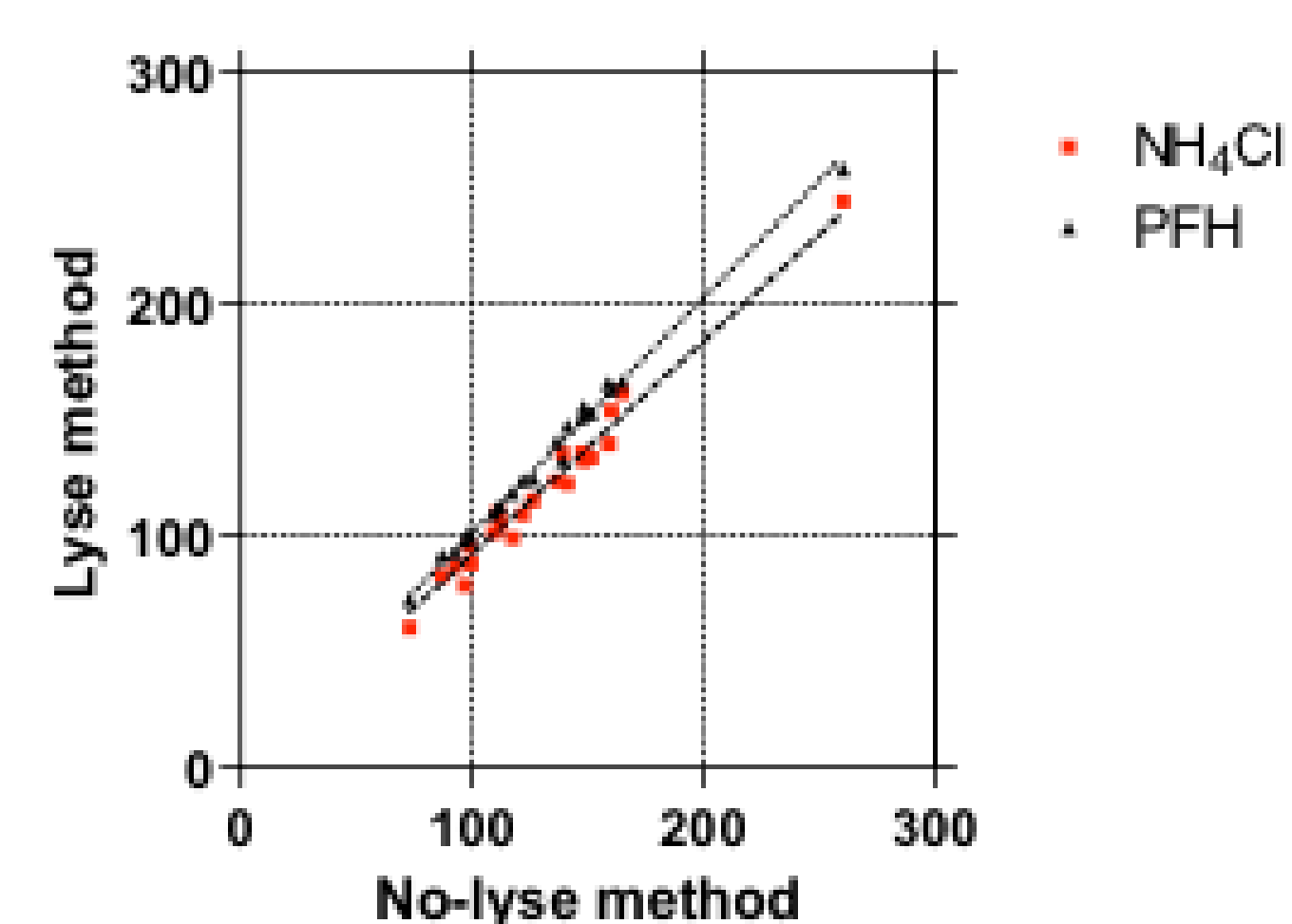

Figure 4. Pairwise comparsions of two lysing methods with respect the no-lyse no-wash approach. Calculations of the linear slope (regression analysis) using non-fixative (NH_4Cl) and PFH-fixative (CyLyse) based methods for red cell lysing, with no-lyse no-wash methods. Nucleated cells were stained with DCV, and DCV-H vs. DCV-A plots were used for doublet discrimination.

CONCLUSIONS

The more conventional way of dealing with doublets is based on scatter signals. However, fluorescent triggering using DNA viable stains may increase single-cell discrimination. Moreover, fluorescence thresholding is typically required at higher sample concentrations needed for large numbers of cells per sample.

Relationships between extinction pulse widths, peak heights, and integrals, tend to be different for symmetric and asymmetric cells, and for single particles and doublets or multiplets, making the discrimination of pathological large cells from doublets and/or aggregates difficult.

Specific DNA fluorescent labeling, can be used to rise above RBC background through fluorescence thresholding, even using lyse-no wash methods.

Scatter for fluorescence parameters chosen for thresholds in a no-lyse no-wash assay should produce the highest separation possible from the background.

At higher sample concentrations/sample input rates, erythrocytes are frequently coincident with leukocytes, but leukocytes are very rarely coincident with each other. Height parameters are more accurate than area due to contributions to the area from these erythrocytes, when no-lyse no-wash methods are used.

Despite limitations, no-lyse no-wash assays are simple and fast and perhaps most importantly, they can minimize sample manipulations and consequent artifacts relevant to the biology of increasingly important assays that target fragile cell subsets and or combine live cell function with cell phenotype.

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