Journal of Immunological Methods 470 (2019) 59–63

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical Note

A novel one-step extracellular staining for flow cytometry: Proof-of-concept on sepsis-related biomarkers

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ARTICLE INFO

Keywords:
One-step protocol
Whole blood analysis
Flow cytometry
Sepsis
CD64
HLA-DR

ABSTRACT

Background: Flow cytometry is a powerful analytical technique. However, it requires time-consuming, multi-step sample procedure. A new protocol was developed to perform extracellular staining and red blood cell lysis in one step, using dry antibodies. Common markers of white blood cells as well as sepsis biomarkers were tested as a model for modulated antigen expression.

Methods: Peripheral blood was stained using the reference and the one-step methods. Recruitment and staining of CD3-, CD4-, CD8-, CD14-, and CD15-positive cells were analyzed. Then, protocol modifications were tested for optimization. Finally, the one-step method was evaluated on subjects in septic conditions, by measuring expressions of CD64 and of HLA-DR.

Results: No statistical differences were observed between methods when comparing the proportions of cells. The procedure was optimized by decreasing blood volume from 100 µL to 5 µL, lysis from 1 mL to 500 µL, and time from 30 to 15 min. In the blood samples from septic subjects, an increase of CD64 on neutrophils and a decrease of HLA-DR on monocytes were observed.

Conclusions: The one-step method, described here-in, enables an accurate, streamlined flow cytometry sample preparation protocol. The simplified phenotyping procedure reduces training requirements and could help overcome logistic constraints in many flow cytometry applications.

1. Introduction

Many research fields increasingly use flow cytometry for its diversity of applications (Baumgarth and Roederer, 2000). However, this powerful cellular analysis technique requires time-consuming sample preparation with inherent sources of human variability, even for the comparably simple protocol of extracellular staining. To date, the vast majority of sample preparation for a whole blood extracellular staining consists of three steps according to the manufacturer's recommendations: first, staining of the white blood cells (WBC) with antibodies, then another incubation for lysis of red blood cells (RBC) with an appropriate buffer, and finally an optional washing step to reduce non-specific fluorescence. In addition, several milliliters of anti-coagulated blood need to be sampled, frequently with subsequent transportation of tubes to a central laboratory, consuming hours or days.

In this study, combining extracellular staining and RBC lysis in a "one-pot" procedure was evaluated, without a subsequent washing step, to address the previously mentioned issues.

2. Material and methods

2.1. Comparison between reference and one-step protocols

For the preliminary evaluation at Immunotech (Marseille, France), EDTA blood samples from five healthy volunteers from the Saint Joseph Hospital (Marseille, France) were tested upon informed consent according to the reagent manufacturer's instructions, all from Beckman Coulter (Brea, California, USA): 100 µL of blood was stained and incubated 15 min at room temperature in the dark with anti-CD3-Allophycocyanin (APC) (clone UCHT1), anti-CD4-Phycoerythrin (PE)

Abbreviations: APC, Allophycocyanin; FITC, Fluorescein Isothiocyanate; HV, Healthy Volunteers; KO, Krome Orange; MFI, Mean of Fluorescence Intensity; mHLA-DR, HLA-DR on monocytes; nCD64, CD64 on neutrophils; Opt., Optimization; PE, Phycoerythrin; PBE, Pacific Blue; RBC, Red Blood Cells; WBC, White Blood Cells

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https://doi.org/10.1016/j.jim.2019.05.001
Received 30 January 2019; Received in revised form 25 March 2019; Accepted 8 May 2019
Available online 10 May 2019
0022-1759/

(clone 13B8.2), anti-CD8-Krome Orange (KO) (clone B9.11), anti-CD14-Pacific Blue (PBE) (clone RMO52) and anti-CD15-Fluorescein Isothiocyanate (FITC) (clone 80H5), at their recommended doses. Then, RBC were removed using 1 mL of Versalyse lysing solution, incubating for 15 min at room temperature.

In the one-step procedure, 100 µL of the same whole blood samples were diluted with the same antibody mix and 1 mL of Versalyse, and incubated for 30 min in the dark. Procedure modifications were also evaluated: antibodies were diluted with either 500 µL or 1 mL of Versalyse, and either 5, 50 or 100 µL of whole blood were treated. The samples were incubated for 15 or 30 min in the dark.

2.2. One-step method for CD64 and HLA-DR assessment on sepsis samples

Anti-CD64-PBE (clone 22) or anti-HLA-DR-PBE (clone Immu-357) were carefully titrated in the one-step method Optimization 3, then separately converted into a “glassified” layer at the bottom of each testing tube, using a drying process, called DURA Innovations technology (Beckman Coulter Inc.) (Correia et al., 2018; Pitoiset et al., 2018), to reduce sources of human error and variability.

First, levels of nCD64 and mHLA-DR in this dried formulation were compared between the reference method and the optimized one-step technique at Immunotech on 5 healthy volunteers from the Saint Joseph Hospital (Marseille, France).

Then, for the application of the method at Edouard Herriot Hospital Immunology Laboratory (Lyon, France), leftover EDTA blood samples from 11 subjects with septic conditions, admitted to the surgical Intensive Care Unit in Edouard Herriot Hospital (Lyon, France), were collected within 24 h after admission. The results presented herein belong to the IMMUNOSEPSIS study (registered as NCT02803346 at ClinicalTrials.gov). As a control group, 11 healthy volunteers were included in the study upon informed consent.

When needed, 500 µL of Versalyse and 5 µL of whole blood were added to the DURA tubes, and the samples were incubated for 15 min in the dark.

2.3. Flow cytometric data acquisition and statistical analysis

Data were collected on a 3-laser, 10-color Navios flow cytometer and analyzed using Kaluza Software version 2.1 (Beckman Coulter Inc.). Comparison of quantitative variables was performed using a paired Student *t*-test, and by Anova for more than two groups. Correlations were evaluated using the Pearson's correlation coefficient. The statistical analyzes were performed using Jump software.

3. Results

Leucocytes subsets have been compared between one-step and reference two-step techniques: CD3+ lymphocytes, CD3+ CD4+ lymphocytes, CD3+ CD8+ lymphocytes, CD14+ monocytes and CD15+ neutrophils. Representative plots are shown in Fig. 1 and detailed statistics are given in Supplementary Table 1. No statistical differences between percentages and numbers of positive-gated cells were observed, meaning that results obtained with the reference and one-step methods were equivalent.

Then, antibody concentration was doubled by decreasing blood volume from 100 µL to 50 µL and lysis volume from 1 mL to 500 µL, but retained 30 min of incubation time (optimization 1). Because the new method combines lysis and staining steps, total experiment time was decreased from 30 min to 15 while keeping a two-fold antibody concentration (optimization 2). The protocol was also tested on a whole blood volume of 5 µL (close to that of a finger-stick), keeping the antibodies concentrated two-fold and reducing incubation time to 15 min (optimization 3). The main parameters of variation between the three methods are summarized in Table 1.

Comparing these three incremental optimizations with the one-step method previously assessed, no statistical differences between the percentages of positive-gated cells were observed, meaning that all methods offer equivalent staining results (Table 2). Numbers of cells are given for more informations.

The signal-to-noise ratios for each marker were calculated for each technique and detailed statistics are given in Supplementary Fig. 1.

The optimized one-step technique 3 (5 µL of blood in 500 µL of Versalyse within 15 min) was finally combined with DURA technology to provide a ready-to-use tube, stable at room temperature. The expressions of CD64 on neutrophils (nCD64) and of HLA-DR on monocytes (mHLA-DR) were first compared between the reference and the optimized one-step method 3, and then studied in 11 subjects in septic shock conditions (3 females / 8 males). Briefly, at onset of shock, the mean age was 72 years, mean Simplified Acute Physiology Score (SAPS) II was 59, and the mean Sequential Organ Failure Assessment score was 11. Four patients (36%) died within 28 days. Flow cytometry results are presented in Fig. 2.

When compared to reference protocol, nCD64 and mHLA-DR levels obtained with the one-step method are not significantly different.

When evaluated in blood from subjects under sepsis conditions, nCD64 level showed a significant increase ($p < .001$) while mHLA-DR expression was significantly decreased (p: 0.005) as compared to healthy volunteers.

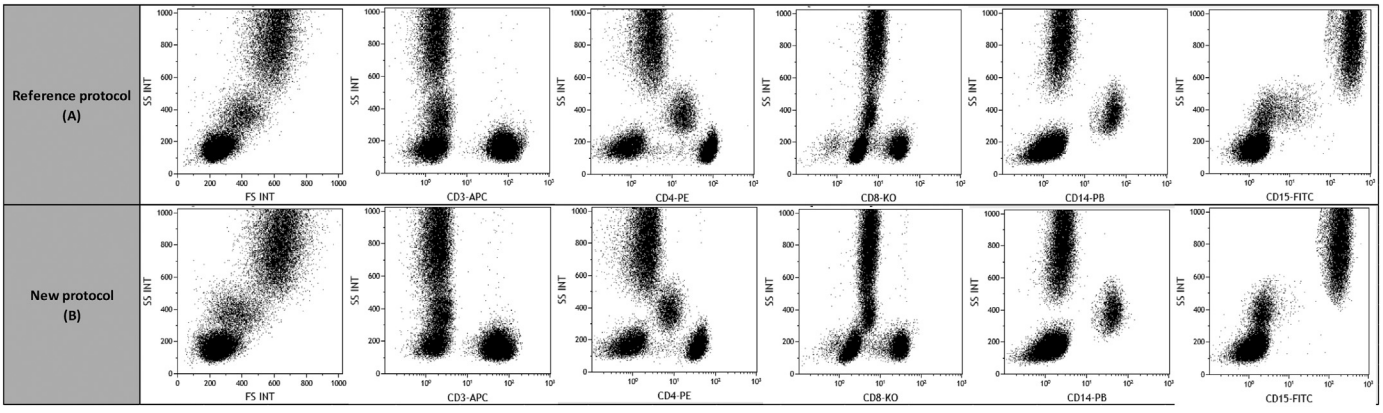

Fig. 1. Analysis of common markers. Representation of the expressions of CD3, CD4, CD8, CD14 and CD15 on leucocytes. Comparison is done between (A) reference protocol and (B) new one-step protocol.

Table 1
Optimizations of the one-step method. Variation parameters are recapitulated for each of the three optimizations (Opt.) in comparison to the one-step method, including lysis volume, blood volume and time of incubation.

	One-step method	One-step method Opt.1	One-step method Opt.2	One-step method Opt.3
Lysis volume (μL)	1000	500	500	500
Blood volume (μL)	100	50	50	5
Time of incubation (minutes)	30	30	15	15

4. Discussion

The study presents a novel one-step method of extracellular staining for flow cytometry. To our knowledge, this is the first time a technique combining WBC staining and RBC lysis has been reported. The aim of the study was to achieve a simpler and faster protocol that may help to democratize use of flow cytometry.

Some authors have already tried to solve the question of having only one step, proposing to remove the lysis step, but necessitating a dedicated flow cytometer (Petriz et al., 2018). The new technique achieved a 15 min one-step sample preparation that reduces overall time required by half, while keeping the important step of lysis, offering a faster extracellular staining protocol for flow cytometry, applicable on any instrument. Some authors have pointed out that cell biology or integrity may be impacted (Breslin et al., 2013); here, the lysis used in the one-step procedure has components that are specifically cleaved by an enzyme uniquely present in RBC membranes, thus that does not affect WBC. Cells of interest are maintained at neutral pH and isotonic conditions.

A good correlation is observed between the percentages of leucocyte subsets gated as positive by the new method and by all of its modifications (increasing antibody concentration, decreasing incubation time and decreasing blood volume), when compared to the traditional reference procedure. No differences in cell numbers were also observed, except, logically, in the procedure using less blood. As expected, signal-to-noise ratios obtained with the tested conjugates show some significant differences between methods, since it depends on each antibody's affinities and concentration. Indeed, in the preliminary evaluation, doses of each antibody were based on manufacturer's recommendations, in order not to introduce a bias between the different methods, but obviously a titration of each antibody in the chosen method could improve the signal-to-noise ratio. This was applied in the second phase of the study to maximize the discrimination of CD64 and HLA-DR antibodies.

Finally, in a proof-of-concept preliminary study, the new optimized method was evaluated with two established biomarkers in the sepsis field and exhibited the expected results: nCD64 level increases in subjects in septic conditions or acute infections (Jämsä et al., 2018), and diminished mHLA-DR is a reliable indicator in research for identifying immunosuppression (Demaret et al., 2013; Monneret and Venet, 2014). Despite an abundant literature, their measurements by flow cytometry do not appear as popular as it should. The new method described here could help fulfill requirements for quantifying such biomarkers with flow cytometry (Dittrich et al., 2016). For instance, using as little as 5 μL of blood could allow use of capillary blood from a fingerstick, reducing the material needed for sampling, and may eliminate time required to transport blood to the laboratory, in case a point-of-care flow cytometer would be available. Using Versalyse and dried conjugates enables room-temperature storage and removes the need for pipetting.

By nature, a proof-of-concept study has limitations: only CD64 and HLA-DR biomarkers were measured; other markers should be assessed

Table 2
Percentages and numbers of positive cells. Mean on five healthy donors of the percentages and numbers of positive gated cells for the one-step method and the three optimizations. Results are presented as mean and Inter Quartile Range between brackets. *P*-values are calculated using ANOVA test, and are considered statistically significant if < 0.05.

	One-step method	One-step method Opt.1	One-step method Opt.2	One-step method Opt.3	ANOVA p-value
CD3+ lymphocytes					
%	30,98 [18,74-49,21]	31,38 [18,78-49,45]	30,66 [18,29-48,4]	29,68 [16,67-46,04]	0,9967
Number	*14,791 [6602–22,390]*	*14,617,2 [6439–22,597]*	*14,226,8 [6237–21,636]*	*1884,8 [813–2781]*	
Among CD3+ cells					
CD4 + CD3+					
%	59,07 [31,37-82,19]	58,7 [30,69-89,39]	58,8 [31,68-82,83]	58,28 [32,11-81,43]	0,9999
Number	*8368,6 [4100–11,608]*	*8213,8 [3845–11,480]*	*7983 [3972–10,991]*	*1050,6 [553–1503]*	
CD8 + CD3+					
%	37,29 [14,87-64,5]	37,93 [15–65,3]	37,46 [14,46-63,81]	38,41 [16,36-63,88]	0,9997
Number	*5875 [982–10,155]*	*5904,2 [966–10,498]*	*5695,4 [902–9840]*	*769,8 [133–1265]*	
CD14+ monocytes					
%	6,42 [3,95-7,72]	6,32 [4,18-7,51]	6,34 [3,84-7,78]	6,53 [4,65-7,68]	0,9955
Number	*3171,4 [1392–4466]*	*3025,4 [1435–4235]*	*3045 [1308–4229]*	*424,4 [227–582]*	
CD15+ neutrophils					
%	48,5 [34,8-60,0]	48,57 [34,61-60,83]	48,62 [35,2-60,59]	49,81 [35,89-60,07]	0,9978
Number	*23,523,8 [15570–33,400]*	*23,060,8 [14435–34,875]*	*23,150,4 [14548–34,988]*	*3262,8 [1941–5032]*	

(A)	Reference method	One-step method	Mean difference	Paired p-value
nCD64 MFI	0,71	0,64	0,078	**0,0897**
	[0,62-0,8]	*[0,52-0,78]*		
mHLA-DR MFI	7,08	6,99	0,092	**0,6154**
	[5,58-8,91]	*[5,43-8,78]*		

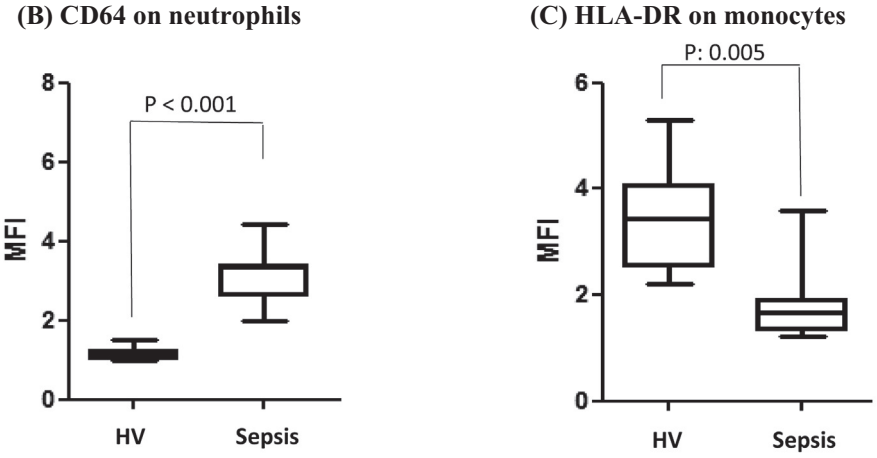

Fig. 2. CD64 and HLA-DR measurements with the optimized one-step method. (A) nCD64 and mHLA-DR MFI comparison on five healthy donors for the optimized one-step method 3 and the reference method. Results are presented as mean and Inter Quartile Range between brackets, with associated mean difference. *P*-values are calculated using a paired Student *t*-test, and are considered statistically significant if < 0.05. (B, C) Comparison of the MFI of nCD64 (B) and mHLA-DR (C) on 11 blood samples from healthy volunteers (HV) and 11 blood samples from subjects in septic conditions (Sepsis). P-values are calculated using a paired Student t-test, and are considered statistically significant if < 0.05.

to underline the global value of the concept. Also, results were reported in a small group of patients; larger studies are needed to assess whether nCD64 and mHLA-DR provide similar expressions when obtained with a one-step protocol.

5. Conclusion

In this study, the easiest protocol of extracellular staining was simplified by simultaneously performing WBC staining and RBC lysis. The method is promising for the cell subsets considered, because it saves time and work while offering discriminative performances similar to the traditional reference protocol. The proof-of-concept of measuring nCD64 and mHLA-DR in subjects in septic conditions has been made. The optimizations achieved suggest potential value of considering this one-step concept for flow cytometric point-of-care applications.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2019.05.001.

Acknowledgements

The authors would like to thank the technician team of the Edouard Herriot Hospital laboratory for their assistance in blood sampling.

Funding

This work was partially supported by Beckman Coulter through donations of laboratory supplies and participation of the two employees mentioned. This private company had no role in the study design, or collection and interpretation of data. Beckman Coulter and the Beckman Coulter product and service marks mentioned herein are trademarks or registered trademarks of Beckman Coulter, Inc. in the United States and other countries. All other trademarks are the property of their respective owners.

Declaration of interest

Fabrice Malergue and Pénélope Bourgoin are Beckman Coulter employees. Pénélope Bourgoin is also recipient of a CIFRE PhD grant n° 2016 / 1368 from the ANRT (National Agency for Research and Technology, FRANCE).

They provided critical advice on setup of the assay and instruments and in manuscript preparation.

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