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TECHNIQUE

Strong impact of the side scatter channel performance in the volumetric flow cytometer CD4+ T cell counts using the CD4% easy count kit

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ABSTRACT

The absolute number and percentage of CD4+ T cells are important parameters in the follow up of HIV-1-infected patients. However, discordant results were found between the CD4+ cell counts and percentage of cells, determined by flow cytometry using the CD4% easy count kit from Sysmex-Partec®. This is a validated kit based on a single-platform methodology for volumetric cytometry counting, and deviations were present despite quality controls were in the acceptable range. Therefore, the photomultiplier performance was analyzed as the possible source of deviation, using pre-counted beads, for the individual channels: side scatter (SSC), fluorescence 1 (FL-1) and FL-2 used in the CD4% easy count kit. Additionally, forward scatter (FSC) and FL-3 were selected for comparison. Then, the coefficients of variation (CV) and accuracy were calculated and beads concentration (count/mL) obtained within the peak of histograms in every optical channel and compared by linear regression analysis. CV, accuracy and linear regression analysis showed fine goodness-of-fit for all channels, but the slope of the SSC was shown statistically different ($p < 0.01$), suggesting bad performance on that channel. Other minor deviations were recorded comparing the intercept of the FSC to FL-1 ($p = 0.03$) and FL-3 ($p = 0.02$). Our results explained previous discordances using the CD4% easy count kit and revealed a previously unappreciated limitation of routine quality controls. They provide new routine quality controls which can be incorporated to detect photomultiplier's error in any flow cytometer even without significant deterioration of performance.

Keywords: flow cytometry, CD4+ T-Lymphocytes, HIV infection, quality control, single platform, CD4+ cell counts

RESUMEN

Impacto significativo del funcionamiento del canal de dispersión lateral en los conteos de células T CD4+ con el uso de CD4% easy count kit. El número absoluto y el porcentaje de células T CD4+ son relevantes para el seguimiento de los pacientes infectados con VIH-1. Sin embargo, resultados discordantes entre ambos parámetros determinados mediante citometría de flujo por el CD4% easy count kit (Sysmex-Partec®) indicaron el posible funcionamiento inadecuado del fotomultiplicador, aun cuando los controles de calidad estaban en el rango aceptable. El kit se basa en una plataforma de metodología única de conteo de células volumétrico. Por lo tanto, se analizó dicho funcionamiento como posible fuente de las desviaciones con cantidades fijas de perlas para los canales de dispersión lateral (SSC), fluorescencia (FL-1) y FL-2. Además, se seleccionó a los canales de dispersión frontal (FSC) y FL-3 como controles, y se calculó el coeficiente de variación (CV) y la precisión. Las concentraciones de las perlas (conteos/mL) bajo el pico del histograma de cada canal óptico se compararon mediante análisis de regresión lineal. Estos tres parámetros mostraron un buen ajuste para todos los canales, pero la pendiente del canal SSC mostró diferencias altamente significativas ($p < 0.01$), lo que sugirió su mal funcionamiento. Se detectó otras desviaciones menores mediante comparación del intercepto del FSC con el FL-1 ($p = 0.03$) y FL-3 ($p = 0.02$). Estos resultados explican discordancias anteriores de este kit y muestra una limitación no detectada de los controles de calidad rutinarios. La metodología propuesta proporciona nuevos controles de calidad a incorporar por el usuario, para detectar posibles errores en los fotomultiplicadores en cualquier citómetro de flujo, incluso sin un deterioro significativo en su funcionamiento.

Palabras clave: citometría de flujo, linfocitos T CD4+, infección por VIH, control de calidad, plataforma única, conteos de células T CD4+

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Introduction

Although WHO's new guidelines to initiate ART reduce the impact of CD4+ T cells counting in the clinical management of HIV-1 seropositive patients [1], its determination is still required in the clinical laboratory to assess the degree of immunodeficiency, to follow up the immune recovery trend and to start antimicrobial prophylaxis when needed [2]. It is a very important matter since even in high income countries like Sweden there are still around 58 % of seropositive cases diagnosed, when they have already less than 350 CD4+ T cells/µL or an AIDS defining condition with very low numbers of CD4+ T cells [3]. The situation is even more critical in low income countries [4].

After the introduction of CD4+ T-cell counting in the follow-up of HIV+ patients, much progress have been achieved up to the development of single-platform absolute determination using 2-color flow cytometry and CD45 gating (pan leucogating) [5]. In

1. WHO, editor. Consolidated guidelines on the use of antiretroviral drugs for treating and preventing hiv infection. Recommendations for a public health approach. 2nd ed. Geneva: WHO Library Cataloguing-in-Publication Data; 2016.

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parallel with the improvements in the methodology, compact size flow cytometers for single-platform volumetric measuring (e.g. Cyflow from Sysmex-Partec GmbH and Auto40 from Apogee Flow Systems Ltd and other similar systems) have been developed to accommodate a higher demand of CD4+ T-cell determinations, in remote areas and at a lower prices [6-9]. Although the performance under field conditions of some instruments have been questioned [10, 11], several studies demonstrated high reliability and precision [6, 7, 12]. However, this equipment requires a skilled operator, the implementation of regular quality control procedures and periodic maintenance to guarantee reliable counting.

In our lab, CD4 counting is determined using the CD4% easy count kit from Sysmex Partec [13]. During exploitation it was noticed that unexpected and discordant results between the CD4+ T cells counts and the percent of same cells arose with some blood samples from HIV+ patients not affected by other comorbidities. Because daily quality controls for laser alignment and volumetric absolute counting resulted in the acceptable range, we hypothesized that perhaps one or more of the photomultipliers involved in the volumetric counting procedure displayed a minor deviation that was influencing the results. To test this hypothesis, we developed a simple methodology to rule out this technical issue.

Materials and methods

Counting Check beads

Count Check Beads (Sysmex, previously Partec, Germany) in bottles with different concentrations of 26 910 and 34 200 particles/mL (± 10 %; low), 99 510 and 100 220 particles/mL (± 10 %; medium) and 218 270 and 242 180 particles/mL (± 10 %, high) for absolute counting quality control using lasers with blue 488 nm emission were used to assess performance of the flow cytometer. Also, other samples of 17 100, 50 110 and 49 755, 121 090 and 109 135 particles/mL were prepared by diluting 1/2 in phosphate-buffered saline (PBS) the previous low, medium and high concentrations, respectively. All dilutions were prepared by a skilled person using a calibrated pipette and the reverse pipetting technique to minimize dilution errors.

Flow cytometer equipment

The CyFlow® space (Sysmex®, Germany) evaluated in this study is a desktop flow cytometer. It is equipped with a 488 nm blue diode pumped solid-state laser and a red laser at 638 nm. Three optical parameters were tested in the study fluorescence 1, 2, 3 (FL1, FL2 and FL3) and photomultiplier for forward scatter (FSC) and side scatter (SSC).

Flow cytometry data acquisition and analysis

The standard layout and settings for Partec® Count Check Beads on the Cytometer were selected. Aliquots of 1 mL of the counting Check beads® at different concentrations were acquired in triplicate until automatic stop to obtain absolute counting and concentration. The Partec® method for True Volumetric Absolute Counting supported by the CyFlow® space is based on the precise measurement of the number of cells or particles in a fixed sample volume by means of two electrodes. During analysis, the sample liquid loses contact first from the upper and then from the lower electrode. These events trigger a ‘start’ and a ‘stop’ signals, respectively, which are stored by software. The volume of sample liquid analyzed between the electrodes is 200 μL. The absolute cell counting, which is based directly on the basic definition of a concentration calculated as c = N/V, where N is the number of cells or particles and V the volume. A precision < 5 % and reproducibility with CV ≤ 2 % are guaranteed by the manufacturer. The analysis was performed for the peak of histograms with Flomax v2.81 software (Partec, Germany).

Statistical methods

To assess the reproducibility, coefficients of variation (CV) were calculated. The accuracy was calculated according to the following formula:

$$\text{Accuracy (\%)} = \left(1 - \frac{|\text{cytometric value} - \text{real value}|}{\text{real value}}\right) \times 100$$

The calculated values were compared using the One-way ANOVA test. Homoscedasticity was verified using the Bartlett’s test.

Beads concentrations (count/mL) obtained within the peak of histograms in the optical channels FL1, FL-2, FL3, FSC and SSC were compared by linear regression analysis. For the independent variable, the concentration of beads was used, whereas, for the dependent variable, the corresponding volumetric counting was assessed. The runs test was calculated to evaluate whether the data differed significantly or not from a straight line. Additionally, pairwise comparisons of slopes and intercepts were evaluated by the F test.

All statistical analyses were carried out with GraphPad Prism software v6.05 (GraphPad Software, La Jolla, CA). A $p < 0.05$ was considered significant (*) and $p < 0.01$ highly significant (**).

Results

Following unexpected discordant results between absolute CD4+ T cells counting and percent of cells, technical errors during sample preparation procedures were ruled out because some processed samples were also acquired in another equipment and results were as expected (data not shown). Because mechanical failures were also excluded, it was decided to test photomultiplier performance in our CyFlow® space cytometer. For this, it was considered that under normal circumstances, volumetric counting in all channels must be the same. Additionally, it would be very improbable that two or three photomultipliers were out of order at the same time. Because the Partec® CD4% easy count kit plots SSC vs FL1 and SSC vs FL2, we tested volumetric counting at different concentrations of beads (in triplicates) in those channels and also in FSC and FL3 for comparison.

Once the values of beads/mL were obtained for every tested channel, the average, standard deviation, coefficient of variation and accuracy was determined. Table 1 and table 2 compile the results for

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Table 1. Raw data of statistical parameters (mean, SD, CV and accuracy (Acc.)) from real bead concentrations as measured by the Sysmex/Partec Cyflow cytometer individual channels before equipment adjustment

Beads/mL (± 10 %)	FSC				SSC				FL-1				FL-2				FL-3			
	Mean	SD	CV	Acc.	Mean	SD	CV	Acc.	Mean	SD	CV	Acc.	Mean	SD	CV	Acc.	Mean	SD	CV	Acc.
17 100	18 022	213	1.18	94.61	16 640	148	0.89	97.31	17 537	215	1.23	97.45	17 705	215	1.21	96.46	17 545	165	0.94	97.40
34 200	35 490	384	1.08	96.23	32 702	397	1.21	95.62	34 547	361	1.05	98.99	34 760	428	1.23	98.36	34 335	334	0.97	99.61
50 100	51 017	1684	3.30	98.19	46 898	1640	3.50	93.59	49 585	1661	3.35	98.95	50 162	1654	3.30	99.90	49 428	1752	3.55	98.64
100 220	99 552	309	0.31	99.33	91 990	296	0.32	91.79	96 885	328	0.34	96.67	97 627	378	0.39	97.41	96 592	288	0.30	96.38
121 090	121 018	859	0.71	99.94	111 900	830	0.74	92.41	117 822	923	0.78	97.30	118 773	804	0.68	98.09	117 375	910	0.78	96.93
242 180	223 937	1530	0.68	92.47	209 112	1348	0.64	86.35	218 202	1540	0.71	90.10	219 992	1486	0.68	90.84	218 163	1510	0.69	90.08

Table 2. Raw data of statistical parameters (mean, SD, CV and accuracy (Acc.)) from real bead concentrations as measured by the Sysmex/Partec Cyflow® space cytometer individual channels after equipment adjustment

Beads/mL (± 10 %)	FSC				SSC				FL-1				FL-2				FL-3			
	Mean	SD	CV	Acc.	Mean	SD	CV	Acc.	Mean	SD	CV	Acc.	Mean	SD	CV	Acc.	Mean	SD	CV	Acc.
26 910	27 602	177	0.64	97.43	27 635	130	0.47	97.31	27 490	169	0.62	97.84	27 435	181	0.66	98.67	27 512	185	0.67	97.76
49 755	53 123	594	1.12	93.23	53 305	541	1.02	92.87	52 950	590	1.11	93.58	52 800	624	1.18	99.31	52 993	596	1.12	93.49
99 510	106 365	1096	1.03	93.11	106 745	1052	0.99	92.73	106 173	1115	1.05	93.30	105 933	1107	1.05	98.46	106 257	1116	1.05	93.22
109 135	116 260	1150	0.99	93.47	116 757	1217	1.04	93.02	116 037	1128	0.97	93.68	115 713	1134	0.98	93.97	116 110	1117	0.96	93.61
218 270	222 278	845	0.38	98.16	223 110	879	0.39	97.78	221 732	859	0.39	98.41	221 263	809	0.37	99.12	221 952	878	0.40	98.31

acquisition of triplicate samples for all the evaluated beads concentrations, before and after equipment adjustment, respectively. Overall, the values of CV ranged from a minimal value of 0.30 to the highest of 3.55. Noteworthy, values higher than 2 % CV were reported for all channels when the concentration of 50 110 beads/mL was tested. Nevertheless, there were not statistically significant differences in these values among the groups ($p > 0.9999$, one way ANOVA). Accuracy values ranged 86.35-99.94 %. Most values were above 90 % except for the SSC channel when the highest beads concentration was tested. Almost all the values obtained in this channel were the lowest for the rest of concentrations tested. Although these observations pointed out to some technical issue, once again the statistical analysis showed not differences among the groups ($p = 0.2009$, one way ANOVA).

These observations prompted us to test whether there was a good correlation between the cytometric counting and the real concentration of beads. As shown in Figure 1A, values obtained with the SSC channel deviated significantly from the boundary of the lines observed for the other channels when the concentration of beads were higher than 100 000 per mL. The regression analysis showed a fine goodness-of-fit for all the channels (Table 3). The coefficient of determination (r^2) was above 0.99 for all the channels and there was no significant deviation from linearity ($p = 0.4000$, runs test). Then, we evaluated whether the slopes and intercepts were significantly different among the regression lines. As displayed in Table 3, all the comparisons between the SSC and the other channels resulted in highly statistically significant differences ($p < 0.01$, F test). Other statistically significant results were observed when the intercept of the FSC was compared to FL-1 ($p = 0.03$, F test) and FL-3 ($p = 0.02$, F test).

In view of the previous unexpected discordant results, we suggested to the technical customer service to check/correct in the first stance the SSC, FSC photomultipliers, and then FL-1 and FL-3. When technical assistance was completed and photomultiplier voltages

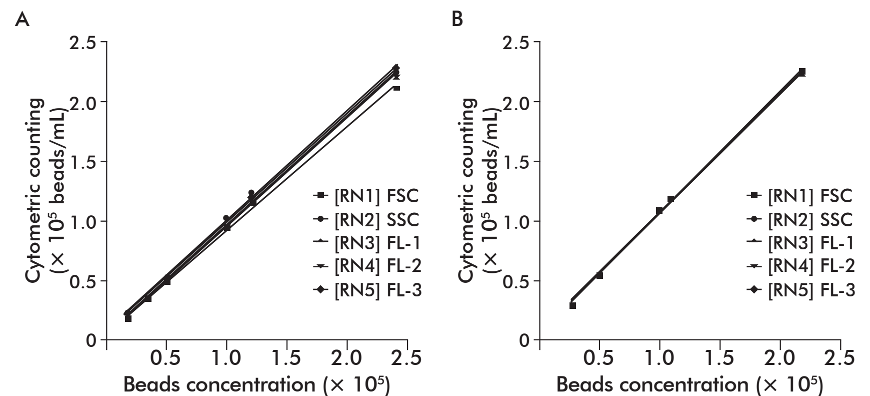

Figure 1. Linear regression analysis of cytometric bead concentrations vs. real bead concentrations for performance determination of the Cyflow® space cytometer. The values were calculated from the mean and SD of three replicate determinations from corresponding individual samples. The full lines represent the best-fit line of each individual channel. A) Before equipment adjustment. B) after equipment adjustment. In the side scatter channel (SSC), the distribution of the values observed is spread out of the remaining regression lines in figure 1A, particularly in the range of 50 000-250 000 beads/mL. Forward scatter (FSC), RN1-5 (Range 1-5), fluorescence 1-3 (FL1-3).

corrected, we observed good agreement between absolute CD4 counts and percent. Next, we underwent a similar evaluation of the cytometer performance.

Representative results for the CV and accuracy are shown in Table 2. Taken together, the values of CV ranged between 0.37 and 1.18 and the accuracy from 92.73 to 99.31. Consistent with prior experiments, the statistical comparison among the channels did not achieve significant differences neither for the CV ($p = 0.9977$, one way ANOVA) nor accuracy ($p = 0.2968$, one way ANOVA). Once again, in an attempt to verify the correlation between the cytometric counting and the real concentration of beads and whether slopes and intercepts obtained in all channels achieved a non-statistical difference among them, a regression analysis was performed. As expected, linear regression analysis revealed a very good determination coefficient value ($r^2 = 0.9988$) and a fine goodness-of-fit ($p = 0.5000$, runs test) for all the channels (Table 4). More importantly, pairwise comparisons showed not

13. WHO. Prequalification of in vitro diagnostics public report. Product: CyFlow® Counter System with CD4 easy count kit and CD4% easy count kit. WHO reference number: PQDx 0350-081-00; Geneva: WHO; 2018.

Table 3. Pair-wise statistical comparisons for the slopes and intercepts of regression lines of Cyflow® space cytometer channels before equipment adjustment¶

Channels	Equation	r^2	FSC		SSC	FL-1		FL-2	
			Slope (p)	Intercept (p)	Slope (p)	Slope (p)	Intercept (p)	Slope (p)	Intercept (p)
FSC	Y = 0.9152X + 5338	0.9981	–	–	–	–	–	–	–
SSC	Y = 0.8557X + 4306	0.9986	<0.0001**	NT	–	–	–	–	–
FL-1	Y = 0.8920X + 5118	0.9982	0.1001	0.0304*	0.0066**	–	–	–	–
FL-2	Y = 0.8991X + 5186	0.9982	0.2498	0.1186	0.0014**	0.6007	0.4656	–	–
FL-3	Y = 0.8919X + 4930	0.9983	0.0923	0.0179*	0.0054**	0.9679	0.8543	0.5912	0.3489

¶ The equation of the best-fit line, the coefficient of determination (r^2) for the goodness-of-fit are shown. The p value (runs test) were 0.4000 for all the channels comparisons.
* Statistically significant. ** Highly statistically significant. NT: It was not tested. The intercept was not tested for channels FL-1 to FL-3 with the SSC channel.

Table 4. Pair-wise statistical comparisons for the slopes and intercepts of regression lines of Cyflow® space cytometer channels after equipment adjustment¶

Channels	Equation	FSC		SSC		FL-1		FL-2	
		Slope (p)	Intercept (p)	Slope (p)	Intercept (p)	Slope (p)	Intercept (p)	Slope (p)	Intercept (p)
FSC	Y = 1.014X + 3031	–	–	–	–	–	–	–	–
SSC	Y = 1.018X + 3004	0.7683	0.6690	–	–	–	–	–	–
FL-1	Y = 1.011X + 3006	0.8731	0.7849	0.6517	0.4907	–	–	–	–
FL-2	Y = 1.009X + 2969	0.7565	0.5827	0.5492	0.3372	0.8795	0.7834	–	–
FL-3	Y = 1.012X + 2991	0.9256	0.8520	0.7067	0.5488	0.9349	0.9000	0.8265	0.7062

¶ The equation of the best-fit line, the coefficient of determination (r^2) and p value (runs test) for the goodness-of-fit were 0.9988 and 0.5000 for all the channels comparisons.

differences among the slopes and intercepts. Thus, as expected from the previous findings, we concluded that lines from the regression analysis were identical (Figure 1B).

Finally, to simulate the impact of the detected errors on performance over for the absolute CD4+ cell counts, the values were calculated for every channel before and after adjustment, based on the equation from the regression line (Table 5). Consistent with prior experiments, errors of values before adjustment in the SSC were higher than or equal to 10 % in all the range tested and the values for the FL-1 were near the 10% limit. After adjustment, error values in all channels were consistently below 10 % as expected.

Discussion

As seen in earlier studies, discordances between CD4+ cell count and CD4 % are common in 16-25 % of HIV-infected patients [14, 15]. Several factors have been associated with this discrepancy like alcohol [16] and steroids [17] consumption, smoking [18], among others. In HIV-1/HCV co-infected patients, discordances between the CD4 % and absolute CD4+ cell counts may increase up to 30 % of the individuals and it is more significant in those with markers of advanced liver disease [19]. In contrast, it is well known that blood samples from infected patients without such co-morbidities CD4+ cell counts and CD4 % show a very good

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Table 5. Estimations of absolute counts of CD4+ T cells and errors based on regression lines for each channel of the Cyflow® space cytometer before and after equipment adjustment¶

ExV (CD4+ T cells/μL)	FSC				SSC				FL-1				FL-2			
	Before		After		Before		After		Before		After		Before		After	
	EsV	E(%)	EsV	E(%)	EsV	E(%)	EsV	E(%)	EsV	E(%)	EsV	E(%)	EsV	E(%)	EsV	E(%)
150	143	5	155	3	133	12	156	4	139	7	155	3	140	7	154	3
200	188	6	206	3	175	12	207	3	184	8	205	3	185	7	205	2
250	234	6	257	3	218	13	258	3	228	9	256	2	230	8	255	2
300	280	7	307	2	261	13	308	3	273	9	306	2	275	8	306	2
350	326	7	358	2	304	13	359	3	317	9	357	2	320	9	356	2
400	371	7	409	2	347	13	410	3	362	10	407	2	365	9	407	2
450	417	7	459	2	389	13	461	2	407	10	458	2	410	9	457	2
500	463	7	510	2	432	14	512	2	451	10	509	2	455	9	507	1
550	509	8	561	2	475	14	563	2	496	10	559	2	500	9	558	1
600	554	8	611	2	518	14	614	2	540	10	610	2	545	9	608	1
650	600	8	662	2	561	14	665	2	585	10	660	2	590	9	659	1
700	646	8	713	2	603	14	716	2	630	10	711	2	635	9	709	1
750	692	8	764	2	646	14	767	2	674	10	761	2	680	9	760	1
800	737	8	814	2	689	14	817	2	719	10	812	1	724	9	810	1
850	783	8	865	2	732	14	868	2	763	10	862	1	769	9	861	1
900	829	8	916	2	774	14	919	2	808	10	913	1	814	10	911	1

¶ . The independent variable is the expected value (ExV; CD4+ cells/μL). Results of absolute counting for every channel (Y) was estimated from the regression lines (estimated value, EsV). E(%): Error percentage calculated as 100 × |1 – EsV/ExV|.

correlation. The data have shown that CD4+ T-cell counts higher than 500 cells/mm^3 are in good agreement with percent of CD4+ T cells above 29 %; for counts of 200-500 cells/mm^3, CD4 % should range 14-28 % and counts below 200 cell/mm^3 with less than 14 % of CD4+ T cells [20].

Reproducibility and comparability of results are important features of good manipulation procedures in flow cytometry. Unfortunately, a survey conducted in UK showed that there were still a lot of cytometry services that did not follow basic laboratory practices such as proper pipetting techniques and instrument maintenance, among other important parameters to ensure compliance with guidelines for CD4+ T-lymphocyte subset enumeration [21]. In this regard, deterioration of cytometer performance may occur progressively and sometimes is not easy to detect it during extensive exploitation in a laboratory with a workload of more than a thousand CD4+ T cells determinations per month. It may happen that photomultiplier performance could behave erratic as a result of a high temperature inside the equipment after hours functioning. It is a common practice in the clinical laboratory to run quality controls at start on every daily working session. Hence, equipment operators could get confused regarding the effect of this phenomenon in the result of CD4+ T cell counting. They might feel that something is going wrong with samples tested after some hours of exploitation or at the end of the day. For instance, they may notice that absolute CD4+ cell counts of some samples from seropositive patients to HIV-1 are not in good agreement with the percent of CD4+ T cells reported by the equipment. In fact, discordant results like 450 CD4+ lymphocytes/mm^3 with 13 % are not expected in patients without any other active infectious diseases, without therapeutic treatments or any other comorbidity that might impact on these parameters, as previously mentioned.

Photomultipliers are long lasting devices which support extensive exploitation with good performance. Even though, they might have some failure as any other electronic device. In the past, it was reported that photomultiplier voltage disparity might be an important source of variability [22]. When failure hinders photomultipliers from working properly, it is immediately notice by the operator. Nevertheless, minor failures are difficult to detect and daily QC controls may still be under the range of acceptance. Such failures can be detected by a skilled engineer after direct verification of the electronic inside the equipment. But laboratory operators cannot perform such procedure.

In our laboratory, we usually use calibration beads and count check beads to assess laser alignment and optimal performance of the cytometer, respectively. However, the findings described in this study, provide evidences that results might be misleading. As shown in the first set of results, there was good laser alignment and recorded data in every channel were on a straight line. Additionally, it must be mentioned that all mean values were in the range of the error of approximately 10 % as reported in the count check bead flasks. Based on those findings, it is considered that there is a proper function of the equipment when it might be not the case.

The results showed in this work, may help to explain previous observations. The procedure of the Sysmex CD4% easy count kit (Code No. 058404) allows absolute counting and percent of CD4+ T cells based on a gate in the SSC vs. CD45-FITC plot that it is further analyzed in a SSC vs. CD4-PE plot [13] (Figure 2). Because of that, bad performance in the SSC has a strong impact on the absolute CD4 counting considering that values in the R1 polygon are affected twice if some error occurs in this channel. However, the impact on the percent of CD4+ T cells is not observed because such systematic error affects values in the R1 and R2 polygons in the same proportion and its negative influence disappears when the ratio R1/R2 is calculated. That is why values of CD4+ T cells percentage were in good agreement with the clinical status of the patients but not the absolute number of CD4+ T cells.

The counting of CD4+ T cells is crucial in the clinical practice for HIV+ patients or those suffering from AIDS [23]. Our report contributes to the present knowledge of quality control methodologies to assess performance in volumetric flow cytometry CD4+ cell counting [24]. A novel, straightforward statistical procedure for testing and compare channel enumeration of check beads is described. It allows verifying individual photomultiplier performance without the need to verify electronic inside the equipment and can be implemented in any laboratory by qualified technical personnel. Moreover, this type of flow cytometry control can be further incorporated as running controls, not only for CD4+ cell determinations, but for any determination made, helping on the monitoring and

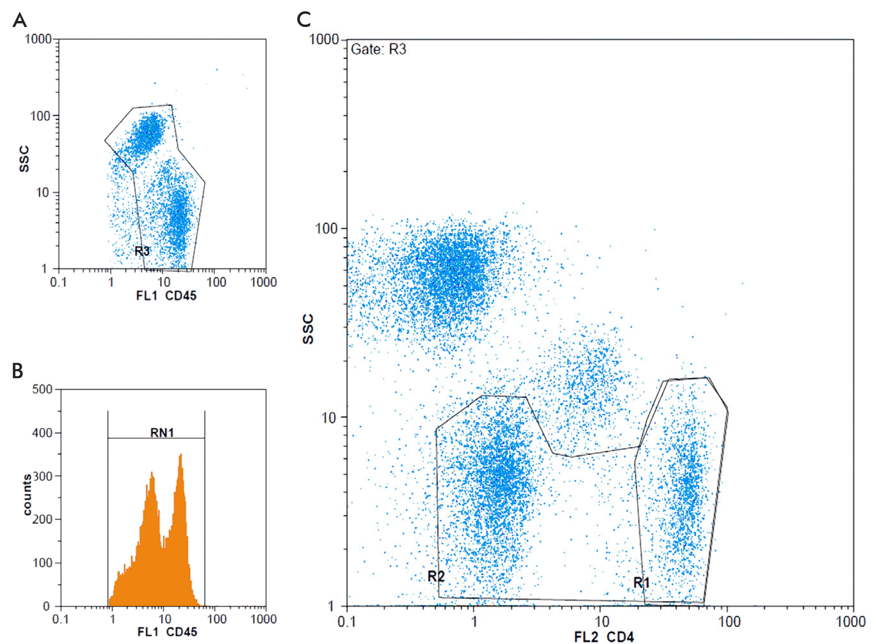

Figure 2. Typical scattergrams obtained with the Cyflow® space cytometer of a stained whole blood sample using the CD4% easy count kit from Sysmex. A) SSC/CD45-FITC dot plot shows all leukocytes populations in R3. B) CD45-FITC histogram representation. C) SSC/CD4-PE shows CD4+ lymphocytes population in R1 and whole lymphocytes in R2. Absolute number of CD4+ T-cells (cells/μL) is calculated as R1 × 0.042 (dilution factor) and the percent as R1/R2.

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technical adjustment of the equipment in addition to periodical service maintenance by equipment manufacturers.

In conclusion, our data converge to indicate that deterioration of cytometry equipment performance may occur progressively without been noticed using standard quality control procedures, and changes in the readings can actually be unrelated to samples. It also illustrates how a minimal deviation in a particular photomultiplier (*e.g.* SSC) might still have an important impact on the final result. In addition to current laboratory QC procedures, our methodology provides additional warranty of good performance. More importantly, it is of general interest for testing equipment performance from the side of the operator working with other flow cytometer or application.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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