

The use of acridine orange and glutaraldehyde-fixed chicken red blood cells for absolute counting of residual white blood cells in leuco-depleted packed red blood cells

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Summary

Background: We have previously developed an affordable flow cytometric method for absolute cell count using glutaraldehyde-fixed chicken red blood cells. However, its use is limited to CD4+ T cells. In the current investigation, we studied the potential use of glutaraldehyde-fixed chicken RBCs to determine the number of residual white blood cells (rWBCs) in WBC-reduced blood component.

Methods: Acridine orange (AO) was used to identify leucocytes in serial diluted blood samples ranging from 0.65 to 1,000 cells/ μ L. The absolute number of AO stained leucocytes were determined by using a known number of glutaraldehyde-fixed chicken RBCs on flow cytometer. The results were compared with the expected value and the absolute count determined by BD Leucocount™ (Becton Dickinson Bioscience). In addition, the stability of AO stained leucocytes and sample stability at various time points were measured. Reproducibility of the assay method was also addressed.

Results: There was a good correlation in the number of leucocytes between our new method and the expected numbers from serially diluted blood samples ($r^2 = 0.99$, $y = 1.04x + 0.50$, $p < 0.001$).

Furthermore, absolute leucocyte counts determined by the new method correlated well with those obtained from BD Leucocount™ ($r^2 = 0.99$, $y = 1.31x - 6.37$, $p < 0.001$). FL-1 intensity and the absolute number of AO stained leucocytes were stable for at least 24 hours after staining. Samples stored at 4 °C were stable for 48 hours and CV of the assay was at an acceptable level.

Conclusion: This flow cytometric method for absolute leucocyte counts using AO and glutaraldehyde-fixed chicken RBCs is a simple, rapid, reliable and inexpensive method for routine monitoring of low levels of leucocytes in blood products. (*Asian Pac J Allergy Immunol* 2012;30:123-9)

Key words: flow cytometry, acridine orange, residual white blood cells, leucocytes, and glutaraldehyde-fixed chicken red blood cells

Abbreviations

A.U.	=	Arbitrary unit
AO	=	Acridine orange
cRBCs	=	Chicken red blood cells
FL	=	Fluorescence
Gm	=	Geometric mean
rWBCs	=	Residual white blood cells

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Introduction

Leucocytes in white blood cell (WBC) reduced-blood components are responsible for a number of well-known side effects in blood transfusion such as HLA-alloimmunization, transmission of infectious agents and transfusion-associated graft versus host disease (TA-GVHD).¹⁻⁴ In order to minimize such transfusion complications, European and America guidelines require the reduction of WBCs in blood



products to a level below 1×10^6 or 3.3 cells/ μL for a 300 mL blood product.⁵ Hence, reliable and sensitive methods for counting a low level of leucocytes are required to assure proper quality control of blood products and to prevent the clinical risk caused by transfusion of leucocytes contaminated blood components.

Different manual and automated methods have been described for assessing low concentrations of leucocytes in blood components. Manual chamber counting using high-volume chambers is a widely used manual technique. However, determination of low levels of leucocytes in blood products using manual methods is not only time consuming but also requires high individual experience and great skill from the person who is performing the cell counting.⁶ Automated counting methods, including the routine hematology analyzer, have been introduced but the primary limitation of this as a low leucocyte counting method is lack of sensitivity.⁷ In order to achieve a good sensitivity and reproducibility for determination of low leucocyte counts in blood components, polymerase chain reaction-based and cellular enumeration by flow cytometry have been applied for this purpose.⁸⁻¹¹ The flow cytometric method using a nucleic dye such as propidium iodine (PI) or 4',6-diamino-2-phenylindole (DAPI) in association with a known number of fluorescent microbeads has been the method of choice for enumeration of residual WBCs in blood components. The absolute number of WBCs is determined by multiplying the cell to microbead ratio by number of beads added. It has been shown that this technique is efficient and the sensitive at concentrations as low as 2 leucocytes/ μL of sample. However, this technique is still limited by the high cost of the currently available fluorescent microbeads.

We have recently reported the use of glutaraldehyde-fixed chicken red blood cells (cRBCs) as a counting bead for CD4 enumeration in the peripheral blood of HIV patients.¹² The results showed a good correlation between absolute CD4+ T cells obtained from the standard microbead method and our cRBCs. Moreover, cRBCs are cheap and easy to prepare. In this study, we used these cRBCs as the counting beads in enumerating the residual WBC counts from leucocytes-reduced blood components and compared these values with those obtained by the standard Becton Dickinson Bioscience (BDB) Leucocount™ Reagent Kit.

Methods

Reagents

Acridine orange (AO) was purchased from Invitrogen, Molecular Probes (Eugene, OR), sheath fluid for flow cytometry and 1xFACSLysing solution from Becton Dickinson Bioscience (BDB, San Jose, CA). The glutaraldehyde-fixed chicken red blood cells were prepared as previously described.¹² Peripheral blood samples were from voluntary donors in the laboratory.

AO staining and flow cytometric gating strategy for enumeration of AO stained leucocytes

One hundred microliters of blood were stained with 30 μL of AO and mixed well with a vortex mixer for 15 sec. The stained samples were then added to 350 μL of 1% FACSLysing solution. After 5 min of incubation in a dark at room temperature (RT), 10 μL of glutaraldehyde fixed-chicken RBCs at a concentration of 134,375-150,109 cells/ μL was introduced into the tube. Finally, the samples were mixed gently prior to acquisition by flow cytometry using FACSCalibur™ with CellQuest™ software (BDB). CaliBRITE™ Beads (BDB) with FACSComp™ software (BDB) were used daily to calibrate the instrument for fluorescence compensation and sensitivity. Data were analyzed with WinMDI version 2.9 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA).

Flow cytometric gating strategies for determination of AO stained leucocytes are shown in Figure 1A. The instrument settings were adjusted to obtain an optimal separation between the AO stained leucocytes and glutaraldehyde-fixed chicken RBCs. The FL1 threshold is set to allow detection of the leucocytes and the glutaraldehyde-fixed cRBCs. A live gate R1 excluding non-nucleated particles, such as platelets, RBCs and debris, was drawn to cover the total leucocyte population. The glutaraldehyde-fixed cRBCs were acquired in R2 based on their FL-4 fluorescent intensity. A minimum of 10,000 events of glutaraldehyde-fixed cRBCs were acquired and the absolute number of leucocytes/ μL was calculated by using the following formula: $(A/B) \times (C/D)$, in which A represents the events of AO-stained leucocytes in gate R1, B is the number of events of glutaraldehyde-fixed cRBCs in gate R2, C depicts the number of glutaraldehyde-fixed cRBCs and D is the sample volume (100 μL).

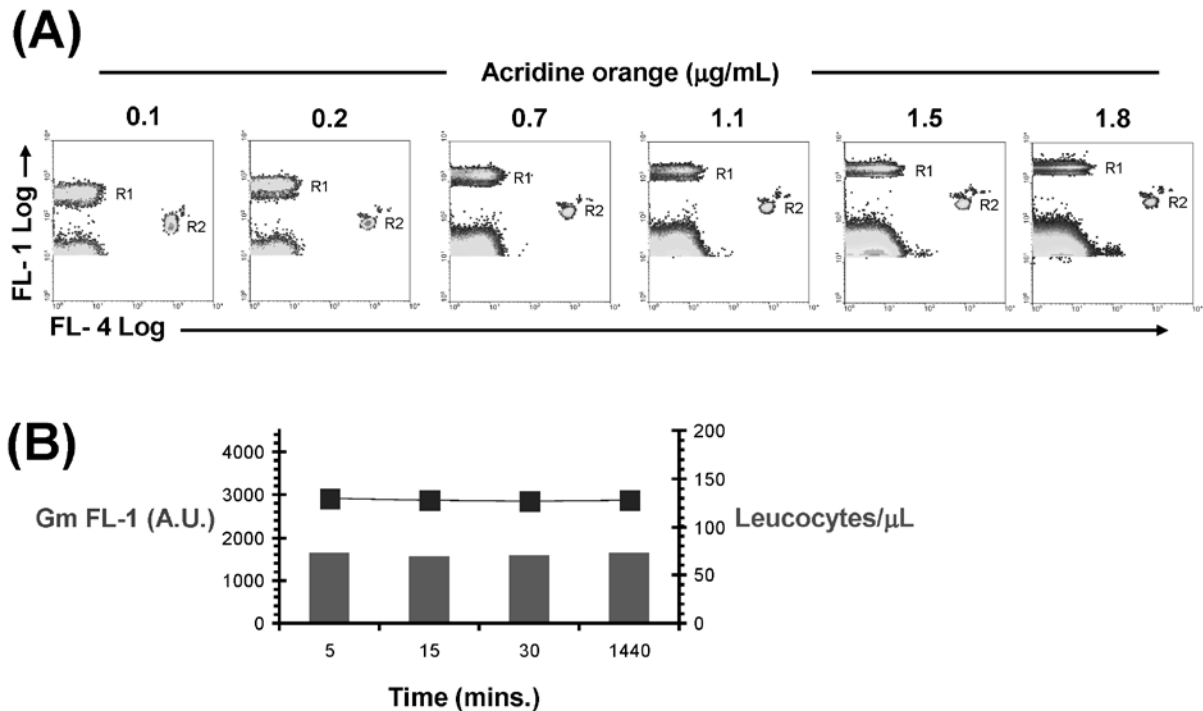


Figure 1. Flow cytometric enumeration of leucocytes by using AO and cRBCs. (A) Representative two-parameter dot plots of FL-4 vs. FL-1 show the AO stained leucocytes and cRBCs in regions R1 and R2 respectively at various concentration of AO. (B) The bar plot shows FL-1 intensity and absolute number of AO stained leucocytes at various time points.

Hematology cell counter

Blood specimens were counted using CELL-DYN 1800™ (Abbott, USA) as a defined reference method. The accuracy of the instrument was regularly checked against its internal standard. The results for the calculated diluted blood samples were considered as expected values.

Counting of residual WBCs by standard bead-based flow cytometric method

The standard BD Leucocount™ Reagent Kit for enumeration of rWBCs in leuco-reduced blood components was used according to the manufacturer's protocol. Briefly, 100 μL of blood specimen was added to the Trucount™ tube (BDB), then 400 μL of Leucocount™ reagent was added. After incubation for 5 min in the dark at RT, data acquisition was performed within 60 min on a FACSCalibur. The instrument setting was also performed according the manufacturer's instructions and a minimum of 10,000 counting of BD Trucount™ beads were acquired. For the analysis of the list mode data, a two parameter dot plot of FL-1 vs. FL-2 gate was set. Leucocytes (R1) and the population of BD Trucount™ beads (R2) were gated

using a sample with a relatively high leucocyte concentration (Figure 2). The absolute number of leucocytes/ μL was calculated with the equation: $(A/B) \times (C/D)$, in which A represents the number of events in leucocyte gate R1, B is the number of events in BD Trucount™ beads gate R2, C depicts the number of beads in the Trucount™ tube and D is the volume of blood sample added (100 μL).

Statistical analysis

Data were collected and analyzed by using Graphpad Prism™ software. Linear regression analysis was used to calculate correlation coefficients and determine the relationship between the different methods. For reporting intra- and inter-assay variability means, standard deviation and coefficients of variation (CVs) were calculated.

Results

Specificity and efficiency of AO for identification of leucocytes in the blood specimens

Experiments were initially conducted to investigate whether the AO is a suitable DNA fluorochrome for staining leucocytes in peripheral blood specimens. The result showed that the AO

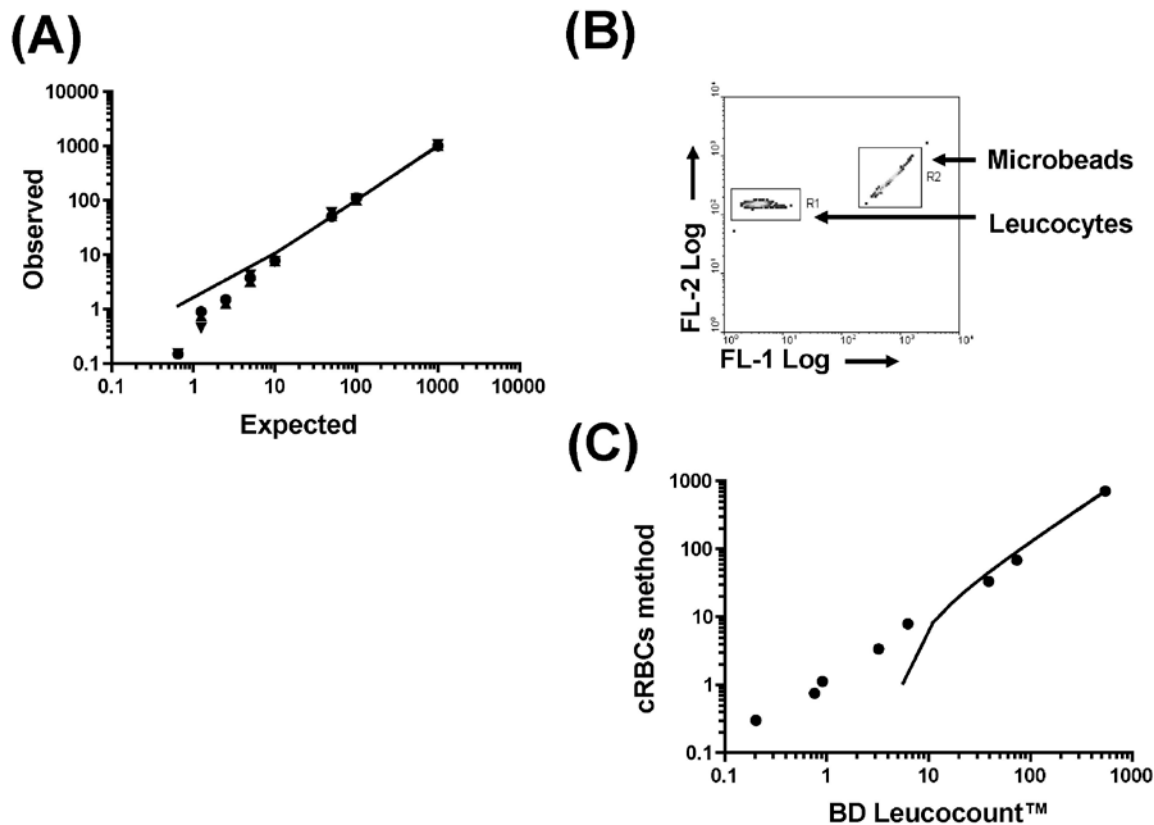


Figure 2. Leucocyte counts using serially diluted whole blood samples. (A) Regression analysis of mean observed absolute leucocytes per μL (y-axis) vs. calculated expected leucocytes per μL according to dilutions (x-axis) measured by the new method. (B) Representative dot plot of FL-1 vs. FL-2 shows the profile of leucocytes in region R1 and Trucount™ beads in region R2 after staining the sample with BD Leucocount™ reagent. (C) Regression analysis of absolute leucocyte counts determined by the new method (y-axis) and the BD Leucocount™ (x-axis).

stained leucocyte population clearly separated from non nucleated cells and debris according to the FL-1 intensities (Figure 1A). In addition, the FL-1 intensity of AO stained leucocytes increased in a dose-dependent fashion and was found to be optimal at a final concentration of 0.7 $\mu\text{g/mL}$ in single FL-1 channel analysis (Figure 1A).

We next investigated the optimum incubation time and the stability of AO stained leucocytes. FL-1 intensity and absolute number of leucocyte in diluted blood samples were determined at 5, 15, 30 min and 24 hour after the staining procedure. Results revealed that the intensity of AO stained leucocytes and their absolute value were found to be optimum at 5 minutes of the incubation time and stable for up to 24 hours as shown in Figure 1B. Taken together, the data indicated the specificity and

the efficiency of AO for staining of leucocyte populations.

Determination of absolute number of AO stained leucocytes by using glutaraldehyde-fixed cRBCs

• **Accuracy and linearity**

We further investigated whether glutaraldehyde-fixed cRBCs can be used to quantitatively determine the absolute number of AO-stained leucocytes and validate whether assessing absolute leucocytes with our new cRBCs method yields comparable results to that of the standard BD Leucocount™. Fresh whole blood samples from healthy volunteers were first investigated for their complete white blood cell count (CBC) then were diluted in phosphate buffered saline (PBS). Each dilution step took place in one step from the fresh blood sample in order to

Table 1. Comparison of rWBC counts obtained by cRBCs method and by BD Leucocount™ in leucodepleted packed RBCs.

Sample no.	cRBCs method		BD Leucocount™	
	events	rWBCs/ μ L	events	rWBCs/ μ L
1	3	0.43	2	0.5
2	0	<0.14	1	0.05
3	1	0.14	3	0.1
4	1	0.15	1	0.05
5	1	0.15	1	0.05
6	0	<0.14	4	0.2
7	1	0.14	1	0.05
8	1	0.14	1	0.05
9	0	<0.14	1	0.05
10	0	<0.14	1	0.05
11	3	0.43	1	0.05
12	1	0.15	1	0.05
13	1	0.15	1	0.05
14	1	0.15	0	<0.05
15	5	0.63	2	0.1
16	1	0.14	1	0.05
17	1	0.14	0	<0.05
18	1	0.14	0	<0.05
19	1	0.14	1	0.05
20	1	0.14	1	0.05

reduce accumulation of dilution errors. Diluted blood specimens were stained with AO and processed as described in the materials and methods section. Absolute leucocyte counts were then determined with our new cRBCs method and the BD Leucocount™. The results showed that the linearity of the leucocyte counts determined by new cRBCs method over a range of 0.65 to 1,000 leucocytes/ μ L. Regression analysis demonstrated a value of $r^2=0.99$ ($y = 1.04x + 0.50$, $p < 0.001$) with the expected value, suggesting a high sensitivity of the novel method to detect a number of leucocytes as low as 0.65 cell/ μ L (Figure 2A). In addition, a good correlation of $r^2 = 0.99$ ($y = 1.31x - 6.37$, $p < 0.001$) was found between the absolute leucocyte counts obtained from the new cRBCs method and BD Leucocount™ (Figure 2B and 2C), Taken together, our data suggest a good linearity and accuracy for absolute leucocyte counts in blood samples.

• Precision and reproducibility

To assess the precision of the single-platform flow cytometric method, the WBC counts from 5 aliquots of the same diluted blood specimen were

assessed for their variability. The data demonstrate a mean variability of 2.4%, 4.0%, 8.5%, 1.8%, 13.0%, 8.4%, 32.8% and 34.8% for 1,000, 100, 50, 10, 5, 2.5, 1.25, 0.65 leucocytes/ μ L, respectively.

• Sample storage stability

Blood samples were tested for their stability by storing them at 4 °C prior to AO staining. Samples were diluted to 10, 5, 2.5, 1.25 and 0.65 cells/ μ L respectively, stained and analyzed at 1, 24 and 48 hours after blood collection. Stabilization was considered acceptable if the WBCs count at 24 and 48 hr. were within 5 to 10 percent of the initial values. Similar results were obtained within 3-7% and 6-10% of initial values, respectively with statistically difference $p > 0.05$.

• Enumeration of leucocytes in leucodepleted packed RBCs

The next experiment was carried out to determine the absolute number of rWBC in leuco-reduced-blood components. Samples from leuco-reduced-packed RBCs obtained by the filtration technique ($n = 20$) were counted. As shown in Table 1, the rWBCs data obtained by using BD Leucocount™ are comparable to data obtained by using our cRBC method.

Discussion

Determination of low levels of leucocytes over the range 0.5-50 cells/ μ L is the critical part for preparation of leuco-reduced blood components.¹¹ Bead-based flow cytometry is the most widely used technique for counting of low numbers of leucocytes in RBC and platelet concentrate. However, this technique is not affordable for all countries due to the high cost of the reference microbeads. Hence, the development of an alternative affordable method is necessary. Recently, we have developed glutaraldehyde fixed cRBCs to quantitate the absolute cell number of CD4 T-cells in HIV-infected patients.¹² However, the use of these beads is limited to CD4+ T-cell counts. In this current investigation, we extend our method for assessing the absolute number of low level leucocytes in blood components.

We have first demonstrated the specificity and efficiency of AO for discrimination of leucocytes from non nucleated cells and debris. Our findings are consistent with other reports using AO for identification of nucleated cells and malaria parasites in whole blood specimens.^{13,14} Our results also show that the FL-1 fluorescence intensity of AO-stained leucocytes was optimum at 5 minutes

after staining and stable for at least 24 hours. This is due to the fact that lysing solution increases the permeability of leucocyte's cell membranes to enable AO staining of DNA in the leucocytes. It is important to note that the minimum volume of lysing solution used in the experiment did not cause the complete lysis of RBCs, suggesting that the fragile leucocytes would not be destroyed. These facts are important for reducing the bias and increasing the accuracy of the assay performance.¹¹ It is arguable that AO staining might affect the auto-fluorescence of cRBCs, but our experiment showed that FL-1 intensity and the absolute count of leucocytes was not affected by AO fluorochrome. Moreover, the intensities of both FL-1 and FL-4 cRBCs were not different over time, suggesting that AO does not affect the auto-fluorescent intensity of cRBCs. Taken together; our data showed the specificity and efficiency of AO for identification of leucocytes in blood specimens. Our experiment also showed that the sensitivity of this new method was similar to that of the BD Leucocount™ method. In addition, there was no difference in the time for staining and acquiring samples.

Leuco-reduction is performed by separation and/or filtration techniques. In order to prevent febrile reaction, less than 5×10^8 leucocytes ($<1,600$ cells/ μL) in 300 mL of leuco-reduced blood components is recommended. Moreover, less than 5×10^6 - 1×10^7 leucocytes (<16 cells/ μL) and less than 1×10^6 leucocytes (<3 cells/ μL) are required in order to minimize the transfer of organisms and to be considered as leucocytes free.^{5,15,16} Our dilution study showed that the glutaraldehyde-fixed cRBCs assay with flow cytometry had excellent correlation with the expected values. It is important to note that the same diluent is used as for the sample when performing the serial dilution experiments. However, it is known that some leucocytes are still to be found after filtration and this could possibly bias the results, leading to higher leucocyte numbers than expected from the dilution itself. Our dilution with PBS showed a good correlation and was significant over the range of leucocytes 0.6 to 1000 cell/ μL . Hence, this is judged reliable in identifying those specimens with leucocytes less than 100 cells/ μL . The reliability and sensitivity of methods for leucocytes enumeration is important for accurate determination of residual leucocytes in leuco-reduced blood components, as this will both require a proper quality control and the maintenance of the established method for each depletion technique and

filter batch. A multicenter evaluation study should be considered for further investigation of the use of our new cRBCs method in determining leucocytes in leuco-reduced blood components.

In conclusion, we used AO in combination with glutaraldehyde-fixed cRBCs for single-platform cell counting of residual WBCs in leuco-reduced blood components; our new method is relatively cheap, for it is less than 6 US\$ compared to 15 US\$ for the standard Leucocount™ kit. The method is simple and reliable with a high sensitivity when counting low levels of leucocytes. Moreover, it can be done on any standard flow cytometer. Therefore, our novel method might be used in routine clinical settings in order to ensure the quality control of the leuco-reduction of blood components.

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