CD45 assisted Pan*Leuco*gating for Accurate, Cost Effective Dual Platform CD4+ T Cell Enumeration.

Deborah Glencross[1], Lesley E. Scott [1], Ilesh V. Jani[2], David Barnett[3] and George Janossy [2]

[1] Department of Molecular Medicine and Hematology, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa,
[2] HIV Immunology, Department of Immunology and Molecular Pathology, Royal Free and University College Medical School, London, NW3, United Kingdom, and
[3] U.K. NEQAS for Leucocyte Immunophenotyping, Royal Hallamshire Hospital, Sheffield, United Kingdom.

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Background: North American and European guidelines for dual-platform (DP) flow cytometry recommend absolute CD4 T cell counts to be calculated from two parameters: the absolute lymphocyte counts obtained on a hematology analyzer and the percentages of CD4+ cells among lymphocytes (CD4%/*lympho*) obtained by flow cytometry. Nevertheless, the identification of lymphocytes is error-prone: a poor match between these common denominators in the two systems is the main source of inaccuracy. In contrast, total leucocyte counts (white cell counts; WCC) and CD4% among the gated CD45+ leucocytes (CD4%/*leuco*) can be determined with greater accuracy.

Methods: We have introduced 'Pan*Leuco*gating', i.e. using total leucocytes as the common denominator for improving the precision of DP absolute CD4 counting. Correlations and Bland-Altman tests were used for statistical analysis.

Results: First, 22 stabilized blood product samples were provided by U.K. NEQAS and a higher accuracy and precision of CD4 counts were documented using Pan*Leuco*gating compared with lymphocyte gating. Next, 183 fresh and 112 fixed (TransFix™) whole blood samples were used to compare DP methods and single platform (SP) methodology, including both volumetric and bead-based techniques. Particularly high correlation and comparable precision of absolute CD4 counts were observed between the SP volumetric method and DP Pan*Leuco*gating (R^2 = 0.990; bias 6 ± SD 17%). The SP volumetric method showed lower levels of agreement with the DP lymphocyte gating (R^2 = 0.758; bias 14 ± SD 51%) and with the SP Bead-based method (R^2 = 0.923; bias 4 ± SD 31%).

Conclusions: These observations show that DP leucocyte counts (WCC) should replace lymphocyte counts as the "common denominator" although CD4%/*lympho* values can, as an extra step, be also readily provided if requested. When coupled with Quality Control for WCC on hematology analyzers, the DP method with CD45 PanLeucogating represents a robust CD4 T cell assay that is as accurate as the SP volumetric technique. This DP method uses only two, CD45 and CD4, antibody reagents and can be run on any pair of hematological analyzer plus flow cytometer.

Key Terms: Blood, CD4 T cell enumeration, Dual platform, Single Platform, Flow cytometry, Hematology analyzer, affordable, cost effective, accuracy, and precision.

With millions of people HIV infected, the AIDS epidemic has placed a crushing burden on the limited health care budgets in developing countries like South Africa. The only flicker of hope is through clinical trials, the introduction of cost effective therapies, and the promotion of vaccination. All of these aims require affordable CD4 counting as part of a service infrastructure. Laboratory protocols for HIV/AIDS disease monitoring, however, follow US and UK based guidelines (1-9), which provide for precise CD4 counting but they are expensive and inappropriate for the developing countries (10-12). Alternative technologies to expensive flow cytometric methods have however, not been widely implemented due to their complexity and limited quality control (14-17). Consequently, making flow cytometry simpler and more affordable is the optimal solution (9-12). During routine CD4 enumeration two different concepts are applied. The first utilizes a dual platform (DP) comprising two instruments viz. a hematology analyzer for absolute lymphocyte counting (ALC) and a flow cytometer for defining the percentage of CD4 cells in a matching lymphoid population, referred to as the 'common denominator'. Absolute CD4 counts are derived from ALC multiplied by CD4% within the "lymphocyte gate" (CD4%/*lympho*) (1–7).

* Correspondence to **Dr D..K. Glencross,**
Department of Molecular Medicine and Hematology, Faculty of Health Sciences, University of the Witwatersrand,Johannesburg PO Box 2551 Houghton, 2041, South Africa.
E-mail: glencross@mighty.co.za
Website: http://www.affordCD4.com

The second concept utilizes a single platform (SP), which includes volumetric counting (12)(Ortho Inc: Cytoron and Partec GmbH: PAS), microfluorimetry (15) (BD Biosciences, San Jose, CA, IMAGN 2000) or the addition of reference beads in known numbers to samples (BD Biosciences, San Jose, CA - Trucount, Beckman Coulter FlowCount beads). DP utilizing lymphocyte gating is still the most widely used strategy for CD4 T cell enumeration, with SP monitoring less widely used (9, 18).

Recent reviews have emphasized the disadvantages of DP compared to SP (9,18-21). In particular, the different methods used by hematology analyzers to generate WCC differentials render lymphocyte counts especially error-prone, and for this parameter no EQA or internal quality control (IQC) is available – a serious disadvantage (20,22-24). Delays in processing (> than 6-12 hours) can result in error of the white blood cell counts but more frequently results in failure to generate an accurate automated differential count on the hematology analyzer (22-25). Further, the match between "lymphocytes" as defined by hematology and by flow cytometry may not be perfect and remains variable depending upon which system is used (5-7,9,10,15,19).

We have recently introduced cost savings into CD4 testing on both DP (11) and SP instruments (10,12,26). Nevertheless, during the implementation of these novel concepts into our routine service, the inaccuracies of absolute CD4 counts observed were clearly mapped into the area of lymphocyte 'mismatch' on DP (15). This problem was clinically important because such errors of lymphocyte counts were particularly frequent in lymphopenic samples (22-24) taken from HIV-positive patients and in those samples that arrived in the laboratory more than 6-12 hours after collection. When the hematological analyzer had "flagged" these samples and labor-intensive manual WCC differentials had to be performed, with high intra-observer variation (22,24,25). Consequently, in this paper we have eliminated the error-prone process of lymphocyte referencing from our laboratory routine practice.

Here we describe the procedure of leucocyte gating or "Pan*Leuco*gating", where CD4+ T cell enumeration is based on the use of all leucocytes (WCC), instead of lymphocytes, as the matching 'common denominator'. A sequential automated gating strategy has been used to include all CD45+ leucocytes and measure the CD4%*leuco*cyte values. In a routine laboratory these steps require only minimal flow cytometry experience, and are especially useful where large numbers of samples are processed. Furthermore, WCC, unlike lymphocyte counts, are the subject of regular Internal Quality Control (IQC) and EQA.

We have also addressed another important factor in accurate CD4 counting: the deterioration of samples due to delays in testing. We used both stabilized blood (27) and fixative (TransFix™, 28-30) to allow the comparison of different CD4 counting methods in expert laboratories.

Accordingly, the aims of our study have been four-fold. Firstly, we have documented the concept of Pan*Leuco*gating *vis-à-vis* lymphocyte gating on DP using 22 long-term stabilized blood products provided by U.K. NEQAS (27). Secondly, we demonstrated, on U.K. NEQAS data that high variations in lymphocyte counting are a regularly finding. Thirdly, on 183 fresh samples 'Pan*Leuco*-' and 'Lymphocyte gating' methods were compared. Finally, 112 samples were stabilized with TransFix™ in order to document the precision and accuracy of absolute Pan*Leuco*gated CD4 counts in comparison with 'state-of-the-art' SP methods, including both volumetric and bead-based techniques.

MATERIALS AND METHODS

Clinical Samples

The study group comprised 20 U.K. NEQAS EQA stabilized blood product samples and 295 K_3EDTA blood samples referred for routine CD4 T cell enumeration by the Johannesburg Hospital and its associated teaching hospitals. The Johannesburg Hospital laboratory, under the current auspices of the South African Institute for Medical Research (SAIMR), has accreditation with the South African National Accreditation Scheme (SANAS), co-ordinates the SAIMR Hematology EQA program and participates both in the U.K. NEQAS external quality assessment (EQA) Immune Monitoring program and the Royal College Pathologists of Australia (RCPA) Hematology Scheme, both that supply stabilized blood products for EQA evaluation. All samples were selected if <6 hours old from the time of collection to ensure accurate white cell counting (22,23,25). From the 295 routine samples 112 had aliquots spared for fixing by adding 100µL TransFix™ in a 1:11 final dilution (26,28-30). One aliquot reached the UK laboratory to perform SP volumetric testing and the second aliquot was analyzed in Johannesburg, both within a 6-day period. Absolute CD4 counts were generated on DP (by L.E.S) using two methods: the Pan*Leuco*gated CD45/4 method (on all samples, Figure 1) and the Lymphocyte gated CD8/4/3 method (all samples) (11) (on 112 samples). The latter 112 samples were also tested by SP technologies: the bead based counting using Trucount® beads (Becton Dickinson Systems; Mountain View, California, USA; by L.E.S in

Johannesburg) and the volumetric method using Ortho Cytoron*Absolute*™ (Ortho Diagnostics Inc. Raritan, New Jersey, USA; by I.V.J in London)

U.K. NEQAS samples evaluation

Over a 2-year period, 20 U.K. NEQAS external quality assessment (EQA) samples of stabilized whole blood (27), received at the Johannesburg Hospital Hematology laboratory, were tested by the routine Lymphocyte gated CD8/4/3 method (11) and by the Pan*Leuco*gated CD45/4 strategy. Only results obtained from the Lymphocyte gated method were reported back for the EQA evaluation as the results of the Pan*Leuco*gated CD45/4 method were regarded to be, at that time, experimental. The results of this experimental Pan*Leuco*gated CD45/4 method were compared with the overall pool means of all the U.K. NEQAS results obtained in >210 laboratories worldwide for both SP and DP technologies.

White Blood Cell (WBC) counting and quality control

Daily appropriate Internal Quality Control to ensure both accuracy and precision of the Beckman Coulter GenS Hematology analyzer (Hialeah, FL) was performed (in Johannesburg). All samples were analyzed once to obtain a white cell count (WCC) and a white blood cell differential (lymphocyte%). On samples that failed an automated differential count (< than 5-10% overall), a manual differential re-count of at least ~100 leucocytes was performed according to the Johannesburg laboratory standard operating procedure for manual differential counts. Absolute Lymphocyte Counts (ALC) were obtained by multiplying WCC by lymphocyte% of the WBC differential. This ALC was then used in the final calculation of the Lymphocyte gated absolute CD4 count. The Pan*Leuco*gated CD45/4 method required only the WCC to calculate absolute CD4 counts. The same WCC value was used to calculate absolute CD4 counts with both the Pan*Leuco*- and Lymphocyte gated techniques.

Antibody panels

In Johannesburg, a triple combination of FITC-conjugated CD45, RD-1 conjugated CD4 and PC5 conjugated CD3 (All Cytostat, Beckman Coulter, BC, Hialeah, FL) was used in the first 20 U.K. NEQAS and 183 fresh samples. In the 112 fresh samples fixed with Transfix™, CD4 (RFT4)-PE was used in combination with CD45-FITC (BC) or with CD45-FITC and CD3-PerCP (Becton Dickinson Systems (BD Biosciences, San Jose, CA), Mountain View, CA). Trichrome antibody reagents (CD8FITC/ CD4RD-1/ CD3PC5; BC) were used in the Lymphocyte gated routine tests. In London, the reagent panels used on the Cytoron included four tubes as per Table 1

Whole blood lysis

All samples prepared in Johannesburg utilized the BC QPrep/Immunoprep whole blood lysis system. The antibodies were titrated for use with lyse-no-wash (QPrep) procedure. Antibodies conjugated with different fluorochromes for combined analysis (see above) were added in a final working volume of 10ì l in 50ì l aliquots of blood. These samples were vortexed once, incubated for 15 minutes in the dark prior to QPrep preparation (1,2) without additional washing steps. In London the samples were lysed using 0.17 M NH_4Cl. The SP bead technology Trucount was used as directed by the supplier.

Flow Cytometry

Daily Internal Quality Control performed on the FACSCalibur (BD Biosciences, San Jose, CA), the XL-MCL (BC) and the Ortho Cytoron*Absolute*™ flow cytometers included linearity and instrument performance checks. In Johannesburg, system performance was verified with stabilized cells (CytoTrol™ BC, monitored through the BC Inter-laboratory Quality Assurance Program) and the U.K. NEQAS Immune Monitoring scheme. Listmode files were stored on all samples in order to retrospectively re-analyze outliers.

Dual platform testing

All samples prepared with QPrep were analyzed within 2 hours on XL-MCL (BC). The routine DP lymphocyte gated method involved using CD3/4/8 staining and an automatic lymphocyte gating incorporating light scatter (1) to generate a percentage of CD3+/CD4+ T cells. In the Pan*Leuco*gated CD45/4 panel, a [CD45 *vs.* SS (complexity)] histogram was used to identify the total white cell population (Gate A; Figure.1, Histogram 1.1). The gated CD45+ leucocytes were displayed in a second histogram (Fig.1, Histogram 1.2) plotting [CD4 *vs.* SS] to determine the percentage of CD4+ lymphocytes within the total leucocyte/ CD45+ population (Gate B; Figure 1, Histogram 1.2). The latter values were used to calculate the Pan*Leuco*gated absolute CD4 cell counts. Typically, when gates in the Pan*Leuco*gated CD45/4 analyses were pre-defined with rectangular gates, a manual intervention was required rarely: <2% of the analyses. A CD4% of lymphocytes was also obtained by identifying the latter within the CD45++ cells (4,8) (Gate C; Figure 1, Histogram 1.1). To calculate the percentage of CD4+ cells within the lymphocyte population (CD4%/*lympho*), the number of events in Gate B (Figure 1, Histogram 1.2) was divided by number of events in Gate C (Figure 1, Histogram 1.1). Again, we emphasize that this

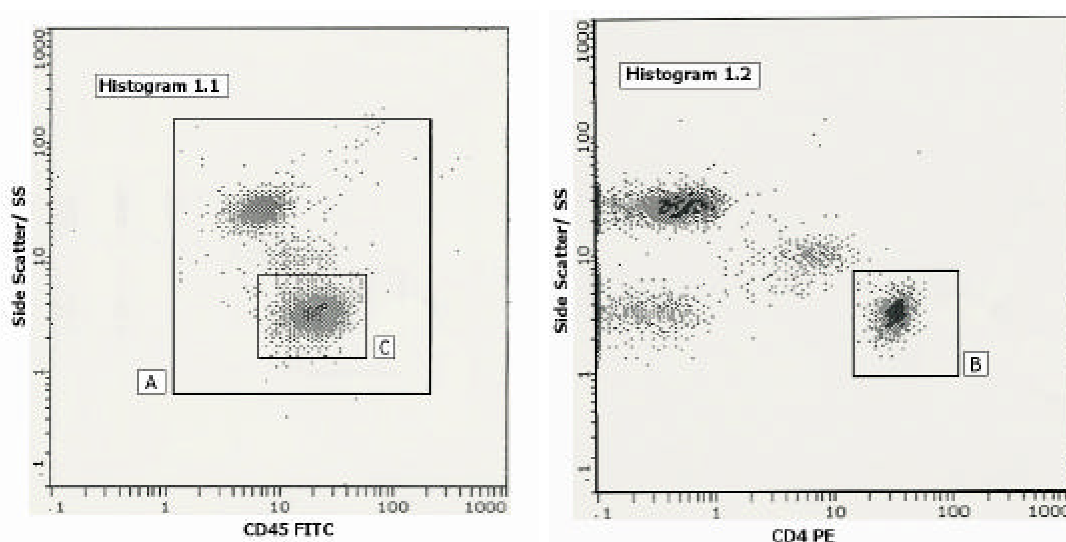

Figure 1. CD4 enumeration by CD45 assisted Pan*Leuco*gating. Total leucocytes are initially identified on a [CD45 *vs.* side scatter (SS)] plot (Region A, Histogram 1.1). All gated events in Region A (Pan*Leuco*cyte) are displayed in a second Histogram 1.2 using a [CD4 *vs.* SS] display where CD4++ lymphoid cells are identified (Region B, Histogram 1.2). In this method, the total leucocytes serve as the 'common denominator' for DP absolute CD4 counting instead of the lymphoid population. This gating strategy also provides for the calculation of a CD4% of lymphocytes, i.e. the number of events in Region B (Histogram 1.2) divided by numbers of events in Region C (Histogram 1.1). This value is however not used in the generation of the Pan*Leuco*gated CD4 counts, and is supplied as extra information when CD4% of lymphocytes may be clinically relevant, e.g. in pediatric cases.

CD4%/*lympho* value was not utilized to calculate absolute CD4 counts. Discrimination from monocytes (Figure 1, Histogram 1.2) was readily achieved due to the high SS and low CD4 expression on monocytes (4,8,12).

Single platform testing

In Johannesburg, Trucount micro-bead analysis was used according to the supplier's recommendations to obtain absolute counts on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). In London, 2 modifications of the volumetric CD4 enumeration were used on the Ortho Cytoron*Absolute*™ (Ortho Diagnostic Systems; ODS, Raritan, New Jersey, USA). First, Ortho-Trio reagents and the Immunocount II software were used in three tubes as described in Table 2. Second, CD45 and CD4 reagents were used in a single tube (***Tube 4***, see Table 2), to mimic the gating strategies of the DP Pan*Leuco*gating (Fig.1) in the volumetric system and to extend previous testing for primary CD4 gating where only a single CD4 reagent was used with a CD4/SS histogram (12).

Statistical analysis.

The evaluation of the results was performed in three steps. Firstly, correlation co-efficients (r) and linear regression analysis between all the methods and sample groups was performed. Secondly, Bland-Altman (BA) plots (31) were used to compare the methods. In this Bland Altman analysis, the SP "gold standard" methods (on the X axis) were plotted against the difference between the "gold standard" SP methods (Volumetric or Bead based) and the DP Lymphocyte gated, the DP Pan*Leuco*gated or the alternative SP CD4 enumeration methods (y axis). The average absolute difference between the two methods, referred to as the bias, as well as the limits of agreements (LOA = mean difference ± 2 standard deviations) were calculated. Thirdly, we expressed the difference between the comparable methods as a percentage of absolute CD4 count. The percentage difference values were plotted vs. frequency on a histogram (Fig. 2). Full agreement between the paired results (i.e. agreement between the two methods) is ideally reflected as 0% difference (or a ratio=1). The advantage of representing the data in this way is that relative differences over a wide range of absolute counts (0->2500 cells/ì l) can be studied. Wide ranges are not readily accommodated by the conventional BA analysis, unless logarithmic conversions are performed (31). A mean of these paired percentage differences (Mean Percentage Difference, MPD), a Standard Deviation of the MPD and a percentage Co-efficient of Variation (%CV) based on the ratio between the corresponding results, was calculated for the whole set of data. With this display, outliers were easily identified and re-investigated to identify factors contributing to aberrant results, without affecting the main body of the data. The percentage differences were plotted on a histogram with a superimposed a "normal" curve to visualize the accuracy and precision of the investigated method in relation to the "gold standard" technique (Fig. 2).

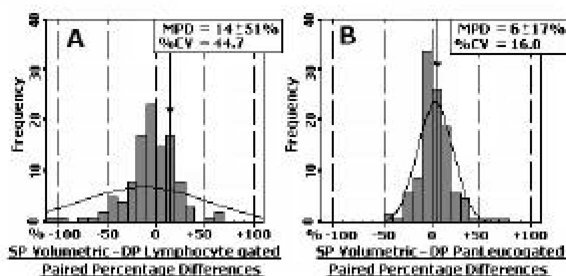

Figure 2.
The paired percentage differences between absolute CD4 T cell results obtained from the "Gold Standard" SP Volumetric with Full Panel and (**A**) DP Lymphocyte gated (N=112) or (**B**) DP Pan*Leuco*gated (n=112). The y-axes represent frequency of the percentage difference; the x-axes represent percentage differences between paired results. The mean of the percentage differences (MPD) reflective of the overall bias between the methods studied, is indicated with a small arrow. The comparative accuracy, i.e. how close the MPD is to 0% difference and the SD of this Mean, as well as the precision i.e. %CV is also shown.
The number of outliers influences the "normal" curve superimposed onto the histogram. No data is excluded and outliers are easily identified.
Abbreviations: MPD = Mean (paired) Percentage Difference; %CV = % Co-efficient of Variation;
DP = Dual Platform; SP = Single platform.

RESULTS

U.K. NEQAS evaluation

Over an 18 month period, 20 stabilized EQA blood product samples were distributed by U.K. NEQAS to >210 laboratories: on average 42% of participants used SP and 58% used DP methods for absolute CD4 counting. The absolute CD4 counts were in the range of 440-1310 cells/ì l; only 2 had <500 cells/ì l. When analyzed in our laboratory by DP Pan*Leuco*gating using CD45/4 staining, the following observations were made.

There was a good correlation between our Pan*Leuco*gated CD45/4 results and the U.K. NEQAS absolute CD4 count overall pool mean based on >210 users (r = 0.963 and R^2 = 0.927; MPD=0±9%; %CV=9.1). Bland Altman analysis demonstrated virtually no bias (-10/ì l; 95% CI +18to -38). Similar good correlations were obtained between our Pan*Leuco*gated absolute CD4 results and the pooled observations of SP users including laboratories using bead-based (r = 0.968 and R^2 = 0.936; MPD=2±7%; %CV=7.1) and volumetric assays (r = 0.934 and R^2 = 0.873; MPD=1±7%; %CV=10.9). Comparison to the pool of DP users showed similar results (r = 0.958 and R^2 = 0.918; MPD=1±9%; %CV=9.0), confirming the validity of our new approach.

U.K. NEQAS results on coefficients of variations for WCC versus lymphocyte counts

The 34 most recent stabilized U.K. NEQAS blood product samples were tested for absolute CD4 counts by DP in 122 laboratories using 26 different types of hematological analyzer. WCC showed a relatively low CV% (Mean 20.0%; range 7.9%-71.9%) whilst absolute lymphocyte counts revealed a high CV% (mean 150.7%; range 34.2%-320.1%). Thus, the difference between the precisions of leucocyte- *versus* lymphocyte counts on hematological analyzers is a general finding between participating laboratories on the U.K. NEQAS Immune Monitoring program.

Comparison of Lymphocyte gating and Pan*Leuco*gating on fresh samples

After considerable initial testing, 183 consecutive fresh samples were analyzed on DP to compare CD45/4 Pan*Leuco*gating with the Lymphocyte gated CD8/4/3 method. The correlation coefficients and linear regression analysis between the methods was acceptable (r = 0.967; R^2 = 0.936). The BA statistics showed relatively wide limits of agreement (+333.3 to -353.3 cells/ì l) with a bias of -10 cell/ì l (95% CI +14.0 to –35.0). Analysis of paired differences expressed as a percentage also suggested some variability between the methods (MPD=4±43%; %CV 41.3). The three outliers noted on the BA analysis were due to the poor forward scatter resolution during Lymphocyte gating.

TransFix™ as a sample stabilizer for inter-laboratory evaluation of Pan*Leuco*gating.

In order to transfer samples between different laboratories for testing, efficient sample stabilization was required. Of the 112 samples treated with TransFix™, good fixation was noted in 109 samples, representing a >97% success rate. Poor fixation and disintegration of polymorphs attributable to fixation artifacts, by morphological and scatter criteria, was seen only in 3 outliers (Figure 3) i.e. samples **2**', **3** ' and **5"**, with pre-fixation these samples showing no evidence of light scatter disintegration. The two other outliers, samples 1 and 4, had other irregularities attributable to absolute cell counts (one each in Johannesburg and London).

Comparison of Pan*Leuco*gating with the 'state-of-the-art' SP technology

The various CD4 counting methods were compared on TransFixed™ samples. During this part of the investigation, the volumetric SP method using the full reagent panel (Table 2) was regarded as the standard predicate technology (Figure 4). As expected, linear regression analysis revealed the best correlation between the two variations of the same SP volumetric method: viz. using the full '3-tube' Trio reagent panel including CD3/4/8 *versus* the simpler '1-tube' CD45/4 protocol (r=0.998 and

R^2 =0.997; y=0.979x+1.9; Figure 4a). A similar excellent correlation was noted when the full Trio panel on SP volumetric was compared to the DP Pan*Leuco*gated CD45/4 method (r = 0.995 and R^2 =0.990; y=0.954x+3.4; Figure 4c).

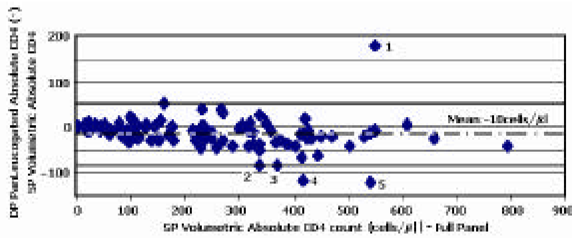

Figure 3. Bland-Altman analysis comparing absolute CD4 counts / µl using TransFixed ™ whole blood.
DP Pan*Leuco*gating and SP volumetric (CytoronAbsolute™ -ODS) were used on the same 112 samples in Johannesburg and London respectively. In order to see a clearer distribution of the results in the lower ranges, only counts < than 800 cells/ì l are shown. No outliers were noted in the range of > than 800 cells/ ì l.

The regression analysis showed a less impressive correlation between the SP volumetric absolute CD4 counts and the SP bead-based CD4 counts using TruCount beads (r = 0.923 and R^2 =0.961; y=0.938x+3.4; Figure 4b). The same correlation has also been depicted with a higher resolution to document that, when compared to the volumetric SP technology, several results obtained by Trucount in the lower CD4 count range were imprecise while such variations were absent among the dataset of Pan*Leuco*gated DP (Figure 5). Finally, as expected from the findings above, the poorest correlations were seen with the DP Lymphocyte gated method versus SP volumetric testing (r=0.871 and R^2=0.758; y=0.610x+70.7; Table 2 and Figure 4d).

Using BA statistics similar observations were made (Table 2). Using the comparison of paired percentage differences, the best agreement was noted between the SP volumetric method using the full panel and its simplified CD45/4 version (MPD=7±=19%; %CV=17.8), closely mirrored by the DP PanLeucogating method (MPD=6±=17%; %CV= 16.0). The SP TruCount system showed a weaker agreement, both in terms of the data spread and the number of outliers (MPD 4±31%; %CV=29.8). Finally, the DP Lymphocyte gating protocol performed especially poorly (MPD=14±51%; %CV=44.7).

During the last part of the investigations the SP bead-based method with TruCount microbeads was used as the predicate/ "gold standard" technology. In this analysis, 11 of the total 112 samples (9.8%) tested were clear outliers by BA analysis and paired percentage difference analysis with the majority in the <400 CD4/ì l range. These outliers were shown to be due to inexperience and poor reverse pipetting technique of the operator (32) and not reflective of the reported precision of bead counting technology itself (33). These 11 samples were therefore removed from the final analysis, which finally comprised 101 samples. The SP TruCount method showed good correlation in this latter analysis with both the SP volumetric protocol (r=0.951and R^2 =0.905; MPD 5±24%; %CV 25.6) and the DP Pan*Leuco*gating method (r=0.940and R^2 =0.884; MPD 1±29%; %CV 28.7). Results of the Bland Altman analysis of the latter are shown in Table 2.

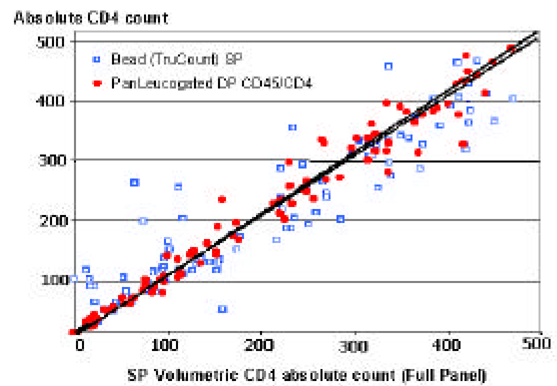

Figure 5. Linear regression analysis of absolute CD4 counts <500/mm³: volumetric SP Cytoron*Absolute*™ (ODS) *versus* CD45/CD4 PanLeucogating protocol (•), and *versus* the bead-based TruCount methods ( ). Note that with PanLeucogating the distribution of individual observation values are tight along the regression line, while with the TruCount methods there is a wider spread, particularly in the range of <150/mm³ CD4 counts.

Finally, as expected from the findings above, the poorest correlations were seen with the DP Lymphocyte gated method versus SP volumetric testing (r=0.871 and R^2=0.758; y=0.610x+70.7; Table 2 and Figure 4d).

Discussion.

A novel, simplified sequential gating system for dual platform CD4 enumeration using CD4, CD45 and SS parameters is described. It avoids the current practice of lymphocyte referencing. In our view, the use of the lymphocyte population as the common denominator between a hematology analyzer and a flow cytometer is not ideal. Instead, emphasis is placed on identifying the CD4+ T cells as a function of a Pan*Leuco*cyte count (or total WCC; Figure 1). By removing lymphocyte matching, with its unacceptably high error rate and wide %CV, the DP cell counting system is greatly improved to reach the precision of the SP methods (7,32,21,20,22,33-36).

The extreme variability of the lymphocyte differentials contributing to poor inter-laboratory %CV's is not a new observation (15,20,22-23). Variability has been documented in both fresh (20,37,38) and stabilized blood products (7,9,39) with up to 40% of the variance between laboratories attributable to differences in analysis (9,20,23,40). The U.K. NEQAS external quality assessment scheme using stabilized blood products reported here, demonstrates a mean %CV of 150.7 for absolute lymphocyte counts in >210 laboratories, as opposed to a mean CV% of 20.0% for WCC, is itself a persuasive argument for abandoning lymphocyte gating.

Theses observations suggest that the various published guidelines for DP CD4 testing may require reconsideration. It has recently been recommended that SP CD4 counting be the method of choice with the lowest documented inter-laboratory CVs of any other method for absolute CD4 counting (9,10,17,20,32). However, the majority of clinical flow-cytometry laboratories worldwide, including U.S.A., still use DP techniques to obtain absolute CD4 counts. The significant cost consequences and technical expertise of SP testing currently restrict the use of such technology especially in under-resourced laboratories, leaving DP technology the simpler, cost effective alternative.

The current guidelines for T cell subset enumeration (1-9) based on lymphocyte referencing may be regarded as inappropriate for the following reasons. Firstly, it is not guaranteed that the same 'lymphoid' population is identified on both the hematology and flow cytometry platforms. Generally they are poorly matched hence the need for extensive guidelines (1-9). Secondly, in order to better match the respective lymphoid population, extra monoclonal reagents are needed to accurately define total lymphocytes by flow cytometry, increasing the costs. Thirdly, quality assurance programs for lymphocyte counts and WBC differentials do not exist because these parameters are difficult to quality control, and therefore their intra- and inter-laboratory %CVs are also generally unknown. Finally, as documented here, 'lymphocyte referencing' and lymphocyte gating can be fully replaced by Pan*Leuco*cyte gating. In contrast to lymphocytes and an absolute lymphocyte counts, white cell counts are well quality controlled with both internal and external quality assessment measurements (U.K. NEQAS, CAP, ACTG, RCPA, etc). Further, we have shown that Pan*Leuco*gating assisted by CD45 is robust and accurate. The PanLeucogating concept is a sequential gating strategy based on side scatter properties of the cells and use of a lineage specific marker, in this instance CD45. White blood cell lineage gating is relatively simple and easy to implement especially in a high output routine setting or where operator experience may limit accurate "bright" CD45 event gating or accurate lymphocyte gating based on light scatter alone. Specific use of the side scatter parameter allows discrimination between CD4 T cells and monocytes for accurate CD4 enumeration (12). Software "autogating" options can be more readily applied to PanLeucogated total CD45+ versus CD45 "bright" events. Further, the challenge associated with accurate pipetting, a requirement for SP with the use of beads, makes the dual platform CD4 testing system incorporating the use of a hematology analyzer derived white blood count, the easier and the better quality controlled option. After all it is the business of the producers of hematology instrumentation to ensure that their instruments produce accurate and precise cell counts. Finally, careful attention to good quality controlled WBC counting can clearly justify the continued use of DP, especially in laboratories that cannot afford SP or who are already making use of hematology analyzers in a routine setting. The need for Good Laboratory Practice, adequate internal quality control and participation in EQA schemes to ensure accurate WCC counting, is also highlighted by the outliers noted in our study. Participation in Hematology EQA schemes, linked with cell enumeration EQA schemes, should be therefore be mandatory in centers that run CD4 T cell counts on DP.

The second important finding of our paper is that, in addition to its primary function of identifying WBC for cell counting purposes (Gate A in Figure 1, Histogram 1.1), Pan*Leuco*gating also serves a number of quality control (7-9) and other clinically relevant functions. Firstly, the use of CD45 facilitates accurate CD4 measurement several days after collection without interfering with PanLeucocyte gating strategies. In our hands (unpublished data) and others (7, 8), we are able to clearly define and easily gate the total CD45 population even after the loss of forward scattering properties, up to 5 days post phlebotomy. Inclusion of the CD45 (5) and SS parameters (4,7,8) avoids technical errors because gating is simple, irrelevant cellular events (red blood cells, platelets) are excluded and relevant lymphoid cells, e.g. with apoptotic scatter features (12), can be included (Table 3). This aspect, together with the successful introduction of fixatives like TransFix™ for preserving both immunological and hematological parameters in whole blood (26,28-30), could exert a great impact on the precision of CD4 counting, particularly in large referral centers and in the developing world (26). Samples can

now be enumerated successfully up to 10-14 days post phlebotomy (Table 2).

The Pan*Leuco*gating approach is particularly amenable to pediatric samples where only small quantities of blood are available. Without CD45 staining, unlysed and nucleated red blood cells may drastically interfere with the definition of both CD4 absolute counts and the CD4%/lymphocyte values. In infants, the CD4%/lymphocyte counts are the clinically relevant parameters because age-dependent variability of WCC render absolute CD4 counts unreliable (Table 3). The use of CD45 facilitates precise CD4%/lympho values by identifying the CD4++ T cells as a function of the bright CD45++ lymphoid cells (CD4+ events contained in Gate B, Figure 1, Histogram 1.1, divided by CD45++ contained in Gate C in Figure 1, Histogram 1.1).

In conclusion, our study, with the assistance of long-term stabilized and short-term "TransFixed™" cells, validates both the DP Pan*Leuco*gating and the SP volumetric system as the most robust, reproducible and accurate flow cytometric assay systems for clinical CD4 enumeration. Of these two approaches, the DP method is already available while the volumetric SP technique still awaits the introduction of simple volumetric flow cytometers (41,42). Interestingly, these are also the systems requiring the least operator input and least expense, and although well suited for resource poor settings (11,12,26,32) may also prove to be of value for cost containment in HIV management in a first world setting.

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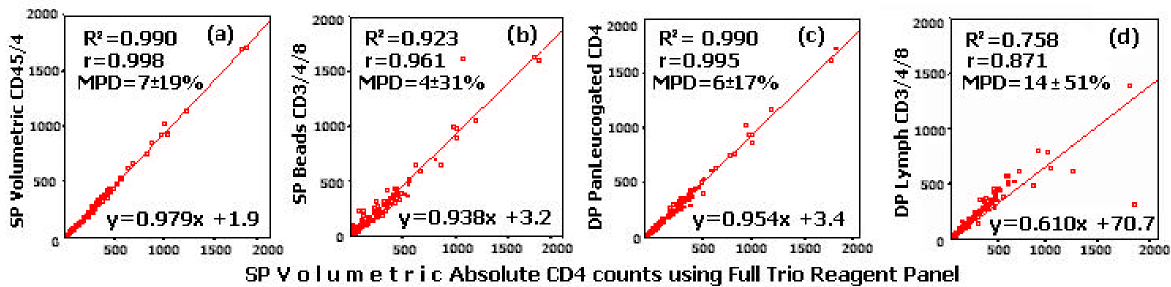

Figure 4. Linear regression analysis on 112 TransFix™ samples. Absolute CD4 counts/µl obtained by SP Volumetric/Full panel (see Table 2 for details) are shown uniformly on all the x axes (**a-d**).
The absolute CD4 values depicted on the y axes are derived as follows: (**a**) SP Volumetric method using CD45/4 staining, (**b**) SP Bead method utilizing TruCount™, (**c**) DP Pan*Leuco*gating using CD45/4 staining, and (**d**) DP Light Scatter based Lymphogating using CD3/4/8staining. The same scales are used on both axes.
Outliers influence the slopes of lines in range of high CD4 counts (in **d**). As expected, the best correlation was noted between the SP Volumetric/Full panel and the same SP method using CD45/CD4 staining, (in **a**). The DP Pan-Leucogating method with CD45/CD4 staining (in **c**) performed equally well.
MPD: Mean Percentage Difference between the methods compared.

Table 1.
Flow cytometric panels performed in London on the Ortho Cytoron*Absolute*.

Full panel (FP)	Tube 1	Isotype controls
	Tube 2	CD4 (OKT4)-FITC/ CD8 (OKT8)-PE/ CD3 (OKT3)-PECY5 (Ortho Trio)
	Tube 3	CD16 (3G8)-FITC/ CD19 (OKB19a)-PE/ CD3 (OKT3)-PE-CY5 (Ortho Trio)
Pan*Leuco*gated CD4	Tube 4	CD45 (Hle-1)-FITC/ CD4 (RFT4)-PE

Table 2.
Bland Altman analysis comparing SP and DP methods of absolute CD4 enumeration.

Methods compared	n	+ve & -ve LOA (Cells/ì l)	Mean (Bias)	95% CI of the Mean
U.K. NEQAS evaluation				
Overall Pool Mean U.K. NEQAS ***vs.*** DP Pan*Leuco*gated CD45/4	20	+110 to -129	-10	+18 to - 38
SP: Bead Pool Mean U.K. NEQAS ***vs.*** DP Pan*Leuco*gated CD45/4	20	+85 to -127	-21.0	+4 to -46
SP: Volumetric Pool Mean U.K. NEQAS vs. DP Pan*Leuco*gated CD45/4	20	+143 to -148	- 2.5	+31 to -36
DP Pool Mean U.K. NEQAS ***vs.*** DP Pan*Leuco*gated CD45/4	20	+109 to -138	-14.5	+14 to -43
TransFix™ evaluation				
SP Volumetric (Full Panel) ***vs.*** **SP: Volumetric CD45/CD4***	112	+46 to -30	+ 8.14	+ 4.6 to +11.7
SP Volumetric (Full Panel) ***vs.*** **SP: Beads***	112	+187 to -157	+15.4	+31.6 to -0.7-
SP Volumetric (Full Panel) ***vs.*** **DP: Pan*Leuco*gated CD45/4***	112	+82 to -49	+16.8	+23.0 to -10.7
SP Volumetric (Full Panel) ***vs.*** **DP: LS Lymphocyte gated CD3/4/8***	112	+368 to -275	+46.2	+76.3 to -16.0
SP Beads CD3/4/8 ***vs.*** **SP: Volumetric Full Panel¶.**	101#	+85 to -121	-17.9	+7.7 to -28.1
SP Beads SP CD3/4/8 ***vs.*** **DP: Pan*Leuco*gated CD45/4¶**	101#	+101 to -107	- 3.4	+6.8 to -13.7

*Bland-Altman (BA) analysis of CD4 counts comparing SP Volumetric method performed on Cytoron*Absolute* (Ortho) using a full panel of Trio reagent *versus* (*vs.)* various other methods. These are: SP Volumetric "Pan*Leuco*gated" CD45/4, SP Beads using Trucount (BD Biosciences) with CD3/4/8, DP "Pan*Leuco*gated" CD45/4 and DP Lymphocyte gated CD3/4/8.

¶A similar comparison is also shown comparing the SP Bead-based method using Trucount with CD3/4/ with all the other methods. Representative examples are shown. #11 outliers were excluded attributable to poor reverse pipetting technique. (Fig.5).

Abbreviations: n = number; +ve = positive; -ve = negative; LOA = Limits of Agreement; CI = Confidence Interval; DP = Dual Platform; SP = Single Platform; LS = Light Scatter.

Table 3 . Clinical significance of CD45 assisted Pan*Leuco*gating

1. **Avoidance of technical errors.**
   a. Quality Assurance and training for WCC procedures in both hematology and immunology laboratories*
   b. Quality Assurance of CD45/CD4 gating strategy
2. **WBC population analysis.**
   a. Definition of all leucocytes (Pan*Leuco*cyte Gate A in Figure 1, Histogram 1.1) as the reference population¶
   b. Definition of CD45++ lymphocytes (lymphoid Gate C in Figure 1, Histogram 1.1) for CD4%/lympho values #
3. **Reliable analysis of blood samples for extended periods.**
   a. Primary CD45 gating prolongs the reliability of subset analysis as CD45/CD4 staining is better preserved than scatter features of WBC subsets (for ~120 hours)
   b. By adding TransFix™ fixative, CD45 gating is extended to 10-14 days
4. **Inclusion of relevant cellular events during analysis.**
   a. Lymphocytes with damaged scatter characteristics but good CD45 staining (in samples taken >16-24 hours earlier)
   b. Apoptotic lymphocytes, frequently seen in infectious diseases such as acute viral infections
5. **Exclusion of irrelevant cellular events from WCC gates.**
   a. Platelet aggregates
   b. Poorly lysed and nucleated red blood cells #
6. **Exclusion of irrelevant cellular events from lymphoid gate.**
   a. Precursor cells expressing low levels of CD45#
   b. Acute leukemic blasts expressing low levels of CD45
   c. Basophils with scatter features of lymphoid cells

* UK NEQAS, QASI, RCPA, CAP.

¶ WCC is used as the 'common denominator' for DP hematological/ flow cytometry CD4 enumeration instead of the lymphocyte population.

Particularly relevant during the analysis of pediatric samples.

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