

Simplified volumetric flow cytometry allows feasible and accurate determination of CD4 T lymphocytes in immunodeficient patients worldwide

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The determination of CD4 cells is of crucial clinical importance for patients with AIDS. However, the high costs involved represent limitations for CD4 cell counting in developing countries. In order to provide an affordable technique, we introduced a simplified volumetric counting (SVC) technique without sample manipulations and investigated it in a multicentre study. Blood samples from 434 healthy donors and immunodeficient patients were tested in eight hospital laboratories in Europe, Africa and Asia. CD4 cell counts were compared using in-house flow cytometric methods and the SVC technique. The SVC method was performed on a low-cost flow cytometer (CyFlow SL, Partec, Münster, Germany) after 15 min antibody incubation without pre-analytic manipulations, such as washing or erythrocyte lysing procedures. Linear regression analysis demonstrated a correlation of

$r=0.942$ (Europe), $r=0.952$ (Africa) and $r=0.989$ (Asia) between the SVC technique and the in-house methods. Bland Altman plot analysis of all patient data showed a mean bias between the two methods of +26 CD4 cells in favour of the SVC technique (measured range: 6–1905 cells/ μ l; median CD4 cell count: 388/ μ l). Three centres used the FACS-count technique (Becton-Dickinson, San José, Calif., USA) as an in-house method dispensing with pre-analytic manipulations. The comparison of SVC and FACS-count method revealed a mean bias of +32 CD4 cells/ μ l (median CD4 cell count: 349/ μ l). The accuracy of the SVC was tested on standards with known CD4 cell counts ($n=6$) and was shown to be 95.2%. The low-cost device and the simplified no-lyse, no-wash test procedure reduces the costs per determination and facilitates the use of flow cytometry in developing countries.

Introduction

In 2002, about 5 million people worldwide became infected with HIV, bringing the total number of persons living with the virus to approximately 42 million. Projections for the next 10 years suggest that the situation will become even more serious, with possibly 100 million infected individuals [1]. About 70% of the HIV-infected persons worldwide reside in sub-Saharan Africa and up to 70% of all hospital beds in African countries are currently occupied by AIDS patients [2,3]. In 2002 alone, about 3.5 million persons were newly infected in sub-Saharan Africa [1]. In

contrast, only 10 000 of about 4 million HIV-infected individuals in South Africa (0.25%) and only 30 individuals out of 1 million infected persons in Malawi (0.003%) can afford access to HIV-related diagnostics and treatment at current prices [4]. This situation not only represents a serious human and ethical catastrophe, but also dramatically diminishes the economic situation in countries with high HIV prevalences [3,5].

Studies have demonstrated that programmes for the affordable diagnosis, treatment and prophylaxis of HIV/AIDS in Africa are highly efficient in improving

the dramatic situation [3,6]. Recently, some pharmaceutical manufacturers have distinctly reduced the prices of their HIV/AIDS drugs in developing countries [3,7]. Therefore, the need for affordable diagnostic tools for the treatment of AIDS in developing countries has also increased.

The determination of CD4 lymphocytes is currently a very important marker of HIV-induced immune impairment [8–10]. It can assess the degree of immune deterioration and speed of progression towards AIDS, and can improve AIDS surveillance through CD4 cell count reporting [11–13]. It can group HIV-seropositive naive patients into cohorts according to their baseline CD4 cell counts before initiating therapy [14]. It can select the optimum timing for prophylaxis of opportunistic infections in AIDS [11,12]. Finally, it can monitor the efficacy of antiretroviral and/or cytokine therapy or protective therapeutic vaccines [15,16]. Therefore, the determination of CD4 cells is of crucial clinical importance for patients with HIV/AIDS [15].

Flow cytometry represents the gold standard for accurate determination of CD4 cells [10]. The high technical and financial expenditure involved in flow cytometric systems and protocols is the main reason for their currently low distribution in developing countries [10,17]. However, the dramatic increase in HIV infections and AIDS in developing countries has led to new approaches aimed at improving this critical situation.

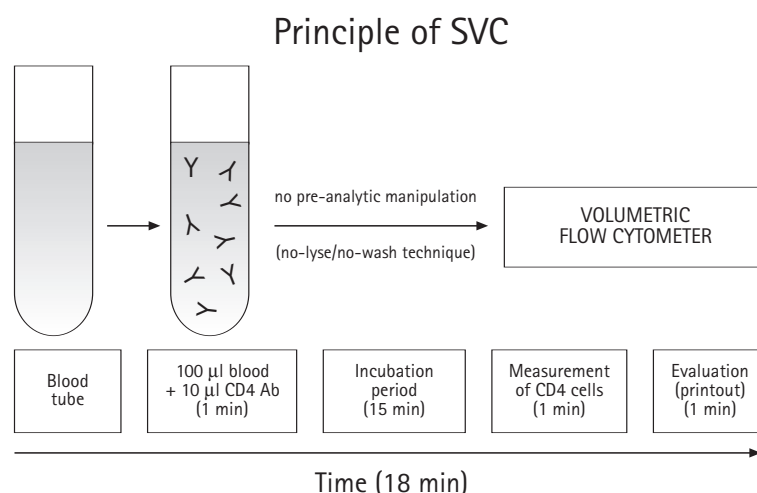
Against this background, we evaluated a new flow cytometric concept for determination of CD4 cells by a highly simplified volumetric counting (SVC) method using a low-cost flow cytometer. A defined blood volume is measured directly after incubation with a single CD4-antibody with no further pre-analytic sample manipulation (Figure 1).

Most flow cytometers do not allow true volumetric counting of cell concentrations by directly measuring cells in given volumes, as is possible with haematology counters. Established flow cytometric protocols for counting leukocyte subsets are often based on a dual-platform analysis (2-PF), taking the percentage of determined leukocyte subsets from the flow cytometric analysis and the total leukocyte counts from a haematology counter in parallel [18]. Studies on 2-PF analyses demonstrated inaccuracies induced by various factors [19–22]. Alternative one-platform (1-PF) protocols added fluorescent beads of known concentrations to the blood samples. The leukocyte concentrations were then calculated in comparison with the defined concentrations of beads [23,24]. Finally, an automated volumetric capillary cytometry system was introduced for determination of CD4 cells [25,26].

A manual microscopic low-cost method for determination of CD4 cells was recently introduced using magnetic beads for selection and quantification of CD4 cells [27]. As a further non-flow cytometric method, the total lymphocyte count was proposed as a simple and cost-effective alternative for therapy initiation in resource-limited settings [28].

However, none of the listed manual and flow cytometric protocols can directly quantify the loss of leukocytes or beads caused by pre-analytic manipulations, such as washing or erythrocyte lysing procedures [18,23,29]. Numerous lysing reagents are available for destruction of erythrocytes in order to achieve good discrimination of target cells [18,30,31]. Erythrocyte lysing procedures have been described as a very critical step in determining leukocyte subsets from whole blood samples [32,33]. Alterations of leukocyte

Figure 1. Principle of the simplified volumetric counting (SVC) method



An aliquot of 100 µl blood is incubated with 10 µl phycoerythrin-labelled CD4 antibody for 15 min and then directly measured in the flow cytometer.

morphology and epitopes or loss of leukocytes have been described when using erythrocyte lysing reagents [29,33,34]. Recently, we demonstrated that a 30–40% pre-analytic loss of leukocytes is caused by all commercial erythrocyte lysing procedures [31].

Our new SVC method includes a new ‘no-lyse, no-wash’ procedure using a single CD4 antibody with subsequent cell counting in a low-cost flow cytometer. This device is the size of a small personal computer and performs a real-time analysis of CD4 cells in a given volume without individual gating strategies.

In order to estimate the feasibility and accuracy of the SVC method, we compared this technique with established flow cytometers and accepted protocols and participated in external proficiency tests. For this purpose, we performed a first multicentre study for determination of CD4 cells by the SVC method, with the collective comprising healthy blood donors and immunodeficient patients at European, African and Asian hospitals.

Materials and methods

Single-centre trial

The sensitivity, precision and linearity of the SVC method were first evaluated in a single-centre trial by performing dilution experiments with known quantities of CD4 cells. Whole blood of healthy donors was leukocyte-depleted using leukocyte filters, and defined concentrations of CD4 cells were added. All determinations were performed with and without a commercial erythrocyte lysing procedure, using the ‘CyFlow’ flow cytometer (CyFlow SL, catalogue number: CY-S-1022, Partec, Muenster, Germany) for SVC as previously described [35].

Multicentre trial

The in-house techniques for determination of CD4 cells were taken as reference methods and compared with the SVC method at eight hospital laboratories. The European laboratories were in Bad Oeynhausen, Berlin, Lübeck and Münster (all in Germany). At these four different centres, one and the same CyFlow device was used (testing period: 1 week, respectively). The African and Asian laboratories were in Douala/Cameroon, Ouagadougou/Burkina Faso, Kigali/Rwanda and Phnom Penh/Cambodia. These centres used different CyFlow devices of the same type. In order to assure the best possible anonymous evaluation, the laboratories were randomly named A–D (Europe) and E–H (Africa and Asia).

All centres prospectively compared their established flow cytometric protocol with the SVC method. Except for one African centre, where the flow cytometric reference method had to be reactivated for this multicentre

trial, all centres performed the reference methods daily for routine determination of CD4 cells.

Inclusion and exclusion criteria

In Germany, blood samples were drawn from healthy blood donors with normal laboratory findings. Blood samples from patients with low concentrations of CD4 cells were drawn either from patients with known HIV or AIDS, or from patients with known malignancies and leukopenia after chemotherapy.

In Africa and Asia, the samples were derived from patients with HIV or AIDS.

Coagulated and old (>24 h) blood samples were excluded.

Sample preparation

Blood samples were collected into EDTA-vials and analysis within 6 h of collection was recommended. The samples were stored at room temperature pending analysis and the vials were placed on a mixing device pending preparation.

The centres were requested to perform both methods in parallel, taking two independent samples from the primary vial at the same time. If the in-house method did not allow both samples to prepare in parallel because of an excessively complex protocol, they were prepared consecutively. The in-house method was performed under routine conditions using the centre’s customary devices, antibodies, reagents and protocols.

SVC method

The technicians working at each centre underwent about 1 h training in the SVC device and protocol prior to the study being started. The determinations with the SVC method and the in-house method were usually performed by one and the same technician, except in Asia, where the reference and SVC method were located at different sites.

Each sample was measured in the 1-PF mode (SVC) using the CyFlow flow cytometer with FloMax software. The CyFlow is equipped with photomultipliers for side scatter (SSC) and only one fluorescence parameter (green solid-state laser with excitation of phycoerythrin at 532 nm). Data acquisition and analysis were performed in real time with a connected notebook computer.

100 µl of blood was incubated with 10 µl of monoclonal CD4 antibody (clone EDU-2, DIATEC, Oslo, Norway) for 15 min at room temperature in the dark. After addition of 2500 µl no-lyse dilution buffer (Partec®, Münster, Germany), the concentrations of CD4 cells were measured in a constant volume of 200 µl samples.

In-house methods

Centre A

Blood samples from 46 healthy donors and from 30 patients with HIV or AIDS were investigated.

The samples were counted in the 2-PF mode using the FACScan flow cytometer (Cellquest software) (Becton Dickinson Biosciences®, San José, Calif., USA) and a haematology counter (K1000, Sysmex®, Kobe, Japan) as the second platform.

The staining was performed with the following antibodies: CD4-PE (clone SK3), CD3-FITC (clone SK7) and CD45-FITC (clone 2D1) (all from Becton Dickinson).

In detail, 100 µl of blood was incubated with 20 µl of each antibody for 15 min at room temperature in the dark. Erythrocytes were then lysed by adding 1400 µl Peli-Lyse buffer lysing solution (1:10 in aqua dest.) (Sanguin Reagents, Amsterdam, the Netherlands) for 10 min in the dark. After 5 min centrifugation (400 g) the cells were resuspended in 1400 µl PBS and centrifuged again for 5 min (400 g). The analysis was performed within 2 h after addition of 400 µl PBS to the cell pellet.

The first tube of each sample was stained with CD45-FITC and was gated on the population of all leukocytes (CD45 cells/SSC). The second tube was stained with CD3-FITC/CD4-PE and gated with identical instrument settings. All CD3/CD4 expressing cells inside the leukocyte gate were defined as true CD4 cells. The total quantity of CD4 cells was determined by calculating the percentage of CD4 cells out of CD45 cells in relation to the concentration of leukocytes in the haematology counter.

Centre B

Blood samples from 30 healthy donors and from 31 patients with leukopenia (after chemotherapy-induced aplasia) and seven patients with HIV/AIDS were investigated.

The samples were counted in the 2-PF mode using the EPICS XL flow cytometer (System II Software) and a Gen-S haematology counter as the second platform (both: Beckman Coulter®, Miami, Fla., USA).

Staining was performed with the following antibodies: CD45-FITC (clone KC56), CD14-PE (clone Mo2), CD3-ECD (clone HIT3a), CD4-PE (clone SFC12T4D11), CD8-FITC (clone AFC121Thy2D8) and isotype controls (all from Beckman Coulter).

In detail, 100 µl of blood was incubated for 20 min with 10 µl of each antibody at room temperature in the dark. The erythrocyte lysis and mild paraformaldehyde fixation were performed using an automatic TQ-Prep device (Beckman Coulter). A three-colour protocol for calculation of CD3/CD4 cell concentrations in the 2-PF mode was used. The concentration of lymphocytes was

determined by three-part differential using a haematology analyser. The flow cytometric analysis consisted of an automatic gating strategy for the lymphocyte population using FSC/SSC and CD14/CD45 dot plots (first tube), excluding contaminating monocytes, and a CD3/CD4/CD8 dot plot (second tube) to quantify the percentage of target cells. The absolute count of CD4 cells/µl was calculated using the leukocyte concentration and the percentage of lymphocytes analysed.

Centre C

Fifty-five blood samples from immunodeficient patients were investigated (25 patients with HIV/AIDS and 30 patients with leukopenia after chemotherapy-induced aplasia).

The samples were counted in the 2-PF mode using a FACScan flow cytometer (Cellquest software) and an LH 755 haematology counter (Beckman Coulter®) as the second platform.

Staining was performed with the following antibodies: CD3-FITC/CD4-PE (clones SK7/SK3) and CD3-FITC/CD8-PE (clones SK7/SK1) (Simultest IMK-lymphocyte kit, Becton Dickinson®).

In detail, a 100 µl blood sample was incubated with 10 µl of each monoclonal antibody for 15 min at room temperature in the dark. Erythrocytes were lysed with BD FACSTM lysing solution (2 ml lysing reagent 1:10 dilution). The samples were centrifuged for 5 min (300 g) and the supernatant was removed. The cell pellet was washed with 2 ml cell wash solution (Becton Dickinson) and centrifuged again for 5 min (300 g). After a final resuspension in 0.5 ml cell wash solution, the cells were measured and evaluated in the 2-PF mode. A gate was set on the lymphocyte population by identifying the FSC/SSC properties. The total numbers of CD3/CD4 cells were determined in relation to the measured lymphocytes in a haematology counter.

Centre D

Blood samples from 30 healthy donors and from 30 patients with HIV/AIDS were investigated.

The samples were counted in the 2-PF mode using an EPICS XL/MCL flow cytometer (System II Software) (Beckman Coulter) and Celldyn 1700 haematology counter (Abbott Laboratories, Ill., USA) as the second platform.

The following antibodies were used: CD45-FITC (clone B3821F4A), CD4-PE (clone SFC12T4D11), CD8-ECD (clone SFC121Thy2D3) and CD3-PC5 (clone UCHT1) (all from Beckman Coulter).

100 µl of each sample was labelled with 5 µl of a four-colour antibody mix for 15 min at room temperature in the dark. Then, 500 µl lysing reagent Optilyse C (Immunotech, Marseille, France) was added to each sample. After another 15-min incubation period in the

dark at room temperature, 500 µl PBS was added to each sample, which was then incubated again for 10 min in the dark at room temperature.

The lymphocyte population among the leukocytes was detected by two different approaches: gating on a CD45/SSC dot plot, followed by detection on an FSC/SSC dot plot. Only cells detected as lymphocytes in both plots were depicted as a CD3/CD4 plot. The percentage of CD3/CD4 cells multiplied by the number of lymphocytes yielded the absolute number of CD4 cells/µl.

Centre E

Ninety-one blood samples from patients with HIV or AIDS were investigated.

The samples were counted with a FACS-count device (Becton Dickinson Biosciences), using the direct, two-colour immunofluorescence method for absolute determination of CD3 cells, CD3/CD4 cells and CD3/CD8 cells.

The kit consists of paired reagent sets with CD3/CD4 (clones SK7 and SK3) and CD3/CD8 (clones SK7 and SK1) antibody-containing tubes with known numbers of fluorochrome-integrated polystyrene beads. The control kit contains fluorochrome-integrated polystyrene beads at four levels.

Briefly, the FACS-count method was performed daily according to the manufacturer's instructions: 50 µl of sample was added to each tube. The tubes were vortexed, and then incubated for 60 min at room temperature in the dark. 50 µl of fixative solution was then added to each tube, which was vortexed prior to being incubated again for 30 min. Finally, the tubes were vortexed and the cells were immediately measured. The system software offers automated analysis, requiring no operator intervention and provides a report quantifying the CD4, CD8 and CD3 cells as absolute numbers and the CD4/CD8 T lymphocyte ratio.

Centre F

Eighteen blood samples from patients with HIV or AIDS were investigated.

The samples were counted in the 1-PF mode using a FACScan flow cytometer in combination with reference (TrueCount) beads (Becton Dickinson Biosciences).

The following antibody combination was used: CD3-FITC/CD4-PE/CD45-PerCP (Becton Dickinson).

50 µl of each sample was filled into a TrueCount tube (BD, ref. 340334) and 20 µl of antibodies were then added. The samples were vortexed prior to incubation at room temperature for 15 min in the dark. Then 450 µl of lysing solution was added and the samples were again incubated for 15 min at room

temperature in the dark. CD4 cells were then measured in relation to the defined concentrations of beads according to the manufacturer's instructions.

Centre G

Forty-five blood samples from patients with HIV or AIDS were investigated.

The samples were counted using a FACS-count device. The procedure was performed as described above (Centre E).

Centre H

Twenty-one blood samples from patients with HIV or AIDS were investigated.

The samples were counted using a FACS-count device. The procedure was performed as described above (Centre E).

Accuracy of the SVC method

The accuracy with which CD4 cells are determined by the SVC method was evaluated with samples from an external proficiency testing programme. The samples and true values were obtained from the German Institute for Standardization and Documentation for Medical Laboratories (INSTAND). For this purpose, six anonymous samples from the German 'immune status' external proficiency test were measured by the SVC method.

Statistical analysis

The reproducibility was analysed after 10-fold determination from the same blood sample and the coefficients of variation (CV) were calculated.

The SVC and reference methods were compared for each centre by linear regression analysis (r). In addition, the mean linear regression was calculated for all determinations from all centres. Furthermore, the mean differences (bias) between SVC and reference methods were calculated for each centre by Bland Altman plot analysis [36]. Finally, the mean CD4 cell count and bias were calculated for all patient data from all centres and from those centres using the FACS-count as their in-house method (centres E, G, H).

The accuracy of the SVC method was evaluated by comparing the desired values and measured values from German external proficiency tests for determination of CD4 cells. The accuracy was calculated according to the formula:

$$\text{Accuracy [\%]} = \left(1 - \frac{|\text{Actual value} - \text{Desired value}|}{\text{Desired value}} \right) \cdot 100$$

Results

The single-centre evaluation demonstrated a linearity of 0.999 in a range between 16 and 568 CD4 cells/ μ l with CVs between 1.7 and 5.5%, as previously described [35].

Multicentre trial

At the European centres, the comparisons of the in-house methods and the SVC technique resulted in correlations of $r=0.936$ ($n=76$), $r=0.968$ ($n=68$), $r=0.978$ ($n=55$) and $r=0.970$ ($n=60$), respectively. Figure 2 demonstrates the regression analysis for the European centres (A–D).

At the African and Asian centres, the comparisons of the in-house methods and SVC technique revealed correlations of $r=0.966$ ($n=91$), $r=0.971$ ($n=18$), $r=0.972$ ($n=45$) and $r=0.989$ ($n=21$), respectively. Figure 3 shows the regression analysis for the African and Asian centres (E–H).

In summary, the comparison of all determinations of CD4 cells from the in-house methods and the SVC technique at all centres yielded a mean correlation of $r=0.944$.

For the European centres A–D, the Bland Altman analysis demonstrated a mean difference (bias) between the SVC technique and the in-house methods of +123, +44, +39 and –68 CD4 cells, respectively.

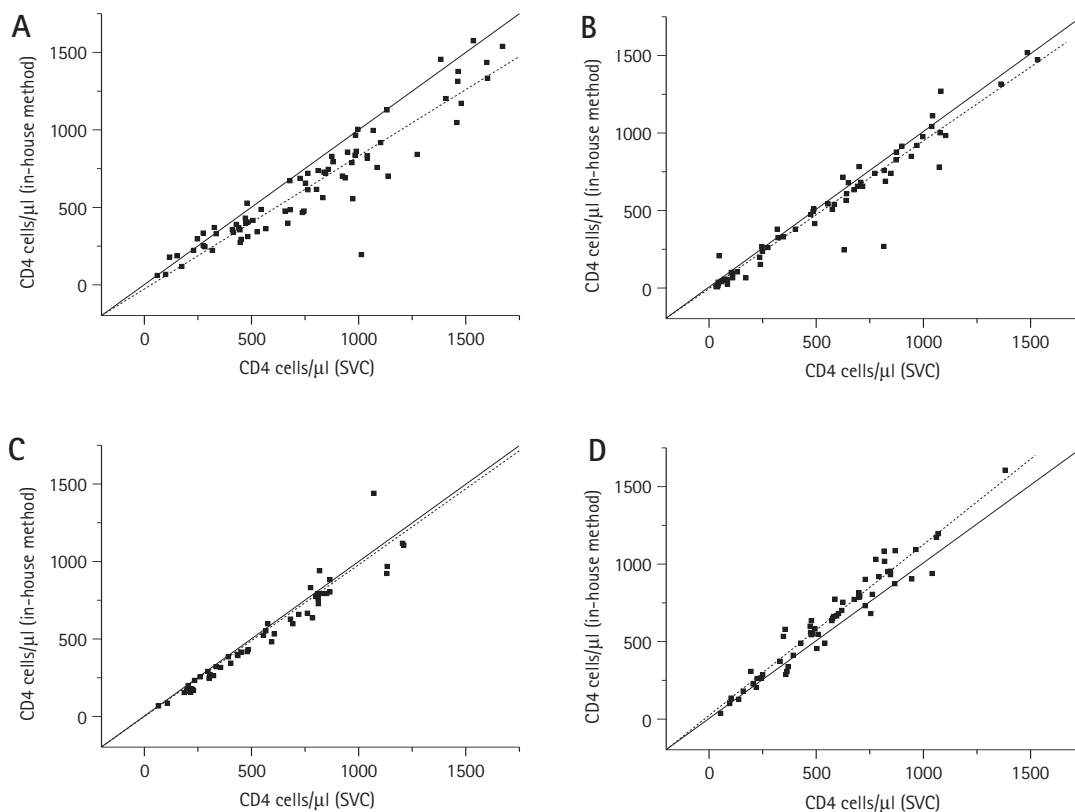
For centres E–H in Africa and Asia, the mean bias of the Bland Altman analysis was +20, –51, +68 and +21 CD4 cells, respectively, between the SVC and the in-house methods.

Consideration of only patient data from all centres (A–H) yielded a mean bias between the two techniques of +26 CD4 cells (median CD4 cell count: 388/ μ l) (Figure 4a).

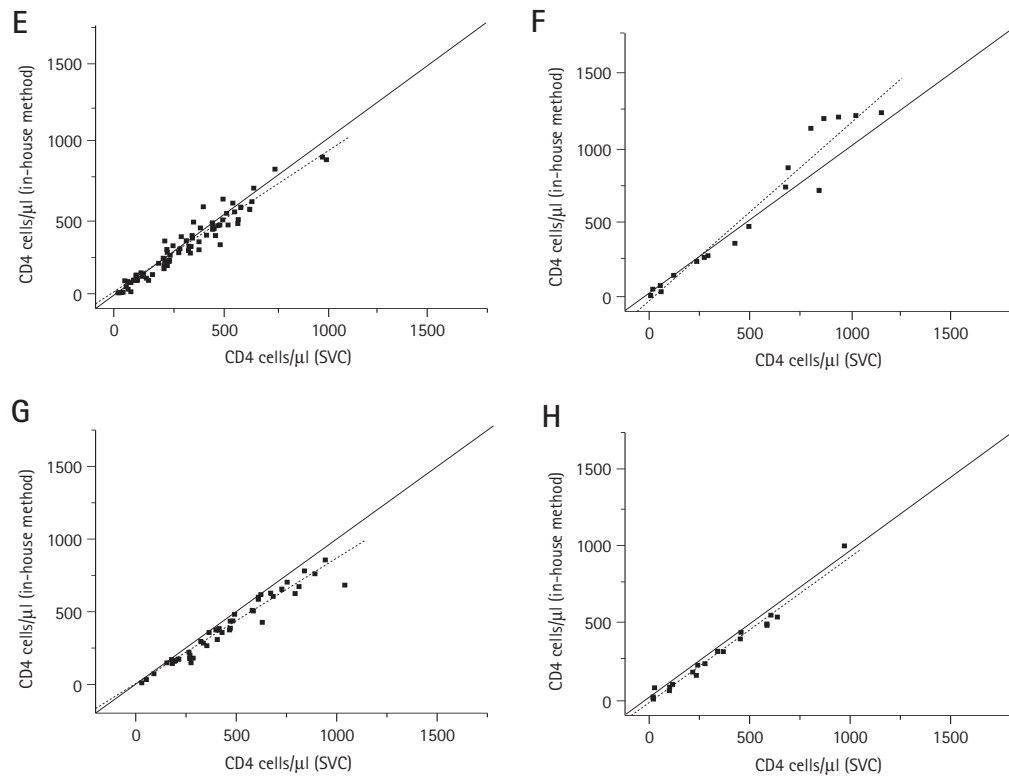
Taking all results from the centres using the automated FACS-count system as their in-house method (centres E, G, H), the Bland Altman plot analysis showed a mean bias of +32 cells (median CD4 cell count: 349/ μ l) (Figure 4b).

Table 1 gives an overview of the median CD4 cell counts and the Bland Altman bias of all centres. At the African and Asian centres, all results derived from patients with HIV/AIDS. At the European centres, the results derived either from healthy donors or from patients with HIV/AIDS or leukopenia. In order to ensure comparability to the African and Asian centres,

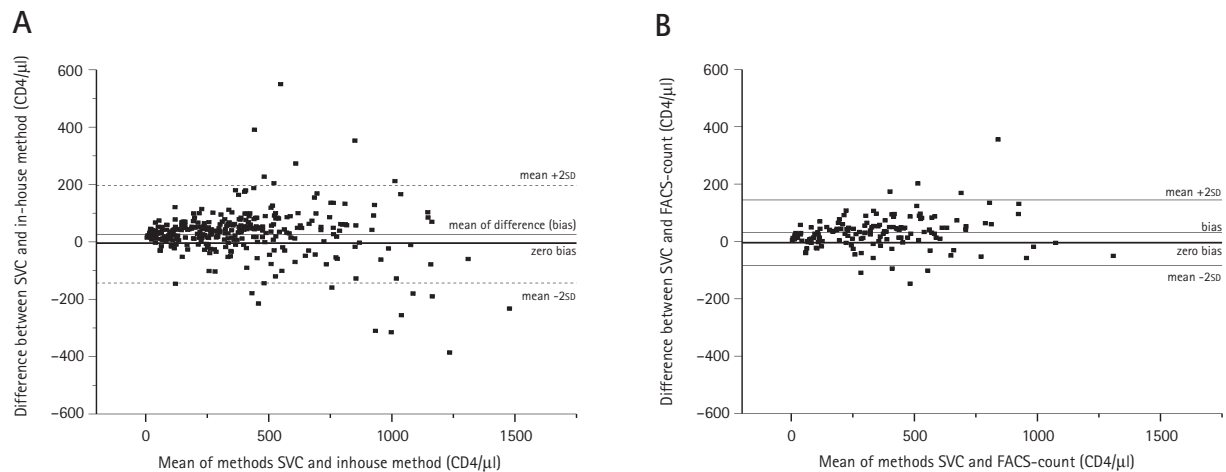
Figure 2. Comparisons between the SVC technique and the in-house methods at the European centres



A–D demonstrate the regression analysis (r) between the two techniques with the theoretical and real coefficient of correlation (full and dotted line) for the European centres (Centre A: $r=0.936$, Centre B: $r=0.968$, Centre C: $r=0.978$, Centre D: $r=0.970$).

Figure 3. Comparisons between the SVC technique and the in-house methods at the African and Asian centres

E-H demonstrate the regression analysis (r) between the two techniques with the theoretical and real coefficient of correlation (full and dotted line) for the individual African and Asian centres (Centre E: $r=0.966$, Centre F: $r=0.971$, Centre G: $r=0.972$, Centre H: $r=0.989$).

Figure 4. Figure 4 shows the mean difference (bias) for the SVC and in-house techniques using the Bland Altman plot analysis with a 95% confidence interval (± 2 sd) (dotted line)

(A) demonstrates a mean bias of +26 CD4 cells for all patients from all centres (A-H). (B) shows a mean bias of +32 CD4 cells when comparing the SVC and FACS-count methods at centres E, G and H.

that group of patients is shown separately in the European centres.

Accuracy of the SVC method

The accuracy of the SVC method for determination of CD4 cells was 95.2% ($\pm 3.3\%$) with a correlation of

0.998 between actual and desired values from six external proficiency tests (Table 2).

Conclusions

The correct determination of CD4 cells is of crucial

Table 1. Median CD4 count and Bland-Altman bias. The results of HIV patients and leukopenia patients are shown separately

Centre	Patients	Number of cases	Median CD4 count (CD4 cells/ μ l)	Bland Altman bias (CD4 cells/ μ l)
A	Total	76	774	123
	HIV	30	385	64
B	Total	68	534	44
	Leukopenia	31	206	33
	HIV	7	616	89
C	Total	55	555	39
	Leukopenia	30	462	34
	HIV	25	658	43
D	Total	60	562	-68
	HIV	30	404	-36
E	HIV	91	324	20
F	HIV	18	494	-51
G	HIV	45	443	68
H	HIV	21	316	21

Table 2. Accuracy of the SVC method related to the true values from external proficiency tests

Sample number	SVC method (CD4 cells/ μ l)	True value* (CD4 cells/ μ l)	Accuracy (%)
1	225	218	96.8
2	1219	1163	95.2
3	92	103	89.3
4	659	622	94.1
5	583	590	98.8
6	591	610	96.9
mean accuracy (%)			95.2
standard deviation (%)			3.30
correlation (<i>r</i>)			0.998

*True value, values from anonymous samples of the German proficiency testing program (INSTAND).

clinical importance for patients infected with HIV or suffering from AIDS. Flow cytometry represents the gold standard for such determinations, but the feasibility of flow cytometric methods in developing countries is controversial [15].

We introduced a new SVC technique for determining CD4 cells, and investigated its feasibility and accuracy in a first multicentre trial at European, African and Asian hospitals. A mean correlation of $r=0.944$ demonstrated a high consistency between the in-house methods and the SVC technique.

The Bland Altman analysis yielded promising results, with a mean bias of +26 CD4 cells for all determinations of samples from patients with HIV/AIDS or leukopenia. The results from those centres using the FACS-count device showed a mean bias of +32 CD4 cells with a median CD4 cell count of 349/ μ l. In comparison to previous studies, the bias obtained in this multicentre study is very satisfactory [18]. The positive bias with slightly higher CD4 cell counts in the SVC method might be due to underestimation of CD4 cells in the in-house methods resulting from erythrocyte lysing procedures, which are known to alter and reduce leukocytes and stem cells during

sample preparation [29,33,34]. Therefore, a comparable no-lyse, no-wash protocol was recently introduced for the similarly important determination of stem cells [37].

In Europe, the SVC method has passed external proficiency tests for determination of CD4 cells with a high accuracy of 95.2% and a correlation of $r=0.998$ (from six samples). We have not yet been able to measure reference samples with defined concentrations of CD4 cells in all countries involved in this study. Nevertheless, these European findings indicate that the SVC method provides accurate values in the enumeration of CD4 cells.

Numerous studies have investigated the intra- and interlaboratory variation for enumeration of CD4 cells using different 1-PF or 2-PF flow cytometric methods, [21,38–41]. Trials comparing the interlaboratory variations between the 1-PF and 2-PF methods for determination of CD4 cells demonstrated a higher interlaboratory reproducibility for the 1-PF techniques [18,21,42]. For the 1-PF methods, studies have reported interlaboratory variations below 5% [18,21,22,42] and a reduction in the interlaboratory CV of about 40% in comparison to the 2-PF technique [21].

The automated 1-PF system FACS-count has been successfully established in many developing countries, since it showed excellent interlaboratory CVs in several studies [18,42–45]. Interestingly, our SVC method demonstrated the highest correlations ($r=0.966\text{--}0.989$) of all centres and a low mean bias using this FACS-count device, which also dispenses with pre-analytic lysing procedures. This high consistency between the automated FACS-count and SVC protocols underlines the reliability and accuracy of both systems. The remarkably good correlation obtained at several centres on different continents may now permit this new, simple and low-cost approach to be extended to the determination of CD4 cells.

In addition to meeting the above-mentioned laboratory standard criteria, flow cytometric systems in developing countries must be affordable and feasible, especially under most unfavourable circumstances. We have therefore evaluated a protocol for determination of CD4 cells without any erythrocyte lysing or washing procedure and direct determination by the SVC device [35].

In this multicentre study, the determination of CD4 cells was performed using only one CD4-PE antibody according to the proposal of Janossy *et al.* [46]. The combination of volumetric flow cytometry and simple protocols with generic monoclonal antibodies has previously been proposed to increase cost efficiency [47]. We have further simplified this trial by introducing a no-lyse, no-wash protocol. Accordingly, aside from the incubation of blood and antibody, the SVC method requires no pre-analytic manipulations (such as erythrocyte lysing or washing procedures). Therefore, it minimizes individual and systematic measurement errors through the constant measurement volumes and the abolition of sample modifications. The introduced no-lyse, no-wash sample preparation protocol in combination with the SVC technique can also be used for counting other leukocyte subsets using phycoerythrin-conjugated antibodies, for example, CD3 or CD8. However, all antibodies have to be used in consecutive measurements.

This approach seems to be advantageous, because a distinct loss of leukocytes due to washing and erythrocyte lysing procedures has been reported by various authors [18,31,35,48]. The same phenomenon was also described for the quantification of stem cells [29,31,33,34], resulting in a recently developed no-lyse, no-wash protocol for determination of CD34 cells [37].

The need for pre-analytic manipulations in nearly all flow cytometric protocols and the high costs of flow cytometric devices are the main reasons underlying the fact that exhaustive determinations of CD4 cells can still not be routinely performed in many developing

countries. Such determinations can be achieved in developing countries only with new techniques and protocols at dramatically reduced costs per sample. Most existing flow cytometric methods suffer from unacceptably high costs and the need for highly developed infrastructures and sophisticated laboratories. This problem has been discussed in various publications, and proposals have been made for improving CD4 cell counting protocols in remote areas at lower costs [17,44,46,49–51].

The costs of the SVC protocol are substantially lower than those of any other flow cytometric method. Using the no-lyse procedure and just one antibody, the total costs per determination, including reagents, disposables and flow cytometer depreciation are between US\$2 and \$3 in most centres. The cost of the SVC flow cytometer is below \$20 000. This sum was achieved by using only two photomultipliers (SSC and FL II) for the determination of CD4 cells. As demonstrated, the one-parameter procedure leads to identical CD4 cell counts, but allows a simpler and more cost-effective measurement [35]. Therefore, both the costs of the SVC device coupled with the usage of a single antibody without further reagents and the personnel expenditure are markedly reduced.

In summary, we have presented a feasible, affordable and accurate flow cytometric method for determination of CD4 cells. It can be performed within a few minutes with no manipulation of samples (except the addition of CD4 antibody) and without complex gating strategies. The SVC method can be performed without experienced laboratory staff, and its deployment is practicable and cost-effective in developing countries, as demonstrated at African and Asian centres. Nevertheless, this technique fulfils the requirements of modern laboratory standards, as shown with reference samples from external proficiency tests. Therefore, larger multicentre studies should be initiated to investigate the benefit of the SVC method.

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Conflict of interest statement

The authors declare no conflict of interest or financial relationships influencing the conclusions of the work.

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