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Simplified Dynabeads Method Using Light Microscopy for Enumerating TCD4+ Lymphocytes in Resource-Limited Settings

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Abstract

Background: We demonstrated the feasibility of implanting the Dynabeads method for CD4+ T lymphocyte enumeration in resource-poor settings (ANRS 1226 study). However, as this technique requires a fluorescence microscope which is not usually available in these settings, WHO has encouraged to simplify the method allowing TCD4+ lymphocyte counting under a light microscope.

Methods: TCD4+ lymphocytes enumeration was assessed using Dynabeads after staining cells nuclei with non-fluorescent dyes and readings under light microscope (DLM). A total of 305 triple of values of CD4 cells counts were generated by both Dynabeads method using a light microscope (DLM), Dynabeads method using a fluorescence microscope (DFM) and the single-platform flow cytometry technique (FCM). The accuracy of DLM was analyzed using 4 fresh blood samples showing 200, 400, 500 and 1000 cells/ μ l in FCM respectively. Correlations have been studied between the 3 methods. The DLM was then evaluated for its ability to correctly segregate absolute TCD4+ lymphocyte values at the thresholds of 200 cells/ μ l and 350 cells/ μ l.

Findings: Cells nuclei staining with Sternheimer-Malbin, Turck1, and Giemsa allows TCD4+ lymphocytes enumeration using DLM. FCM has shown the greatest standard deviations and amplitudes. The reproducibility of DLM was better than FCM. The correlation coefficient between FCM and DFM was 0.975 and it was 0.973, 0.972 and 0.969 with DLM using Sternheimer-Malbin, Turck1 and Giemsa, respectively. The ability of DLM to correctly segregate TCD4+ lymphocyte values at the threshold of 200 cells/ μ l and 350 cells/ μ l was good.

Conclusion: Reliable TCD4+ enumeration can be obtained with DLM. These results will contribute in resource-limited-settings to further reduce the cost of TCD4+ lymphocytes counting and make it more widely available in peripheral laboratories and even in central laboratories that face problems with maintenance and stock-out of reagents for flow cytometers.

Keywords: TCD4+ lymphocytes enumeration, Dynabeads method, Fluorescence microscopy, Light microscopy, Flow Cytometry, Resource-limited settings

Introduction

Human immunodeficiency virus (HIV) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS). In 2012, there were an estimated 2.3 million new HIV infections, 35.3 million people living with HIV of which 71% reside in sub-Saharan Africa, and 1.6 million AIDS patients have died [1]. The disease progression is seen as a decline in TCD4+ lymphocytes counts and an increase in the plasma HIV/RNA viral load [2]. With the introduction of highly active antiretroviral therapy (HAART), the disease progression is slowed and the HIV-infected population have an improve quality of life leading to decrease in morbidity and mortality [3]. However, one cause of concern remains the monitoring of patients on HAART therapy [4]. Monitoring HIV infection progression, time to start prophylactic treatment of opportunistic infections, initiation of HAART and the response to HAART is traditionally carried out using TCD4+ lymphocytes counts and HIV/RNA viral load. Indeed, according to WHO recommendation (http://apps.who.int/iris/bitstream/10665/208825/1/9789241549684_eng.pdf?ua=1) [5], as a priority, antiretroviral therapy (ART) should be initiated in all adults and adolescents with severe or advanced HIV clinical disease (WHO clinical stage 3 or 4), adults and adolescents with a TCD4+ lymphocytes count \leq 350 cells/ μ l. ART should be initiated in all children less than 2 years of age or children younger than 5 years of age with WHO

clinical stage 3 or 4 or CD4 count \leq 750 cells/ μ l and children 5 years of age and older with WHO clinical stage 3 or 4 or CD4 count \leq 350 cells/ μ l. Cotrimoxazole prophylaxis is recommended for adults (including pregnant women) with severe or advanced HIV clinical disease (WHO stage 3 or 4) and/or with a CD4 count \leq 350 cells/ μ l [5] (WHO 2016).

Unfortunately, conventional methods for TCD4+ lymphocytes enumeration require expensive sophisticated equipment usually inaccessible for many areas in middle and low income-countries which carry the majority of the burden of HIV-infected population [2,4,6-8]. Since the poor and economically weak third world nations cannot afford regular CD4+ T cell counts and HIV-1/RNA viral load testing, cheaper and alternative methods for HIV disease progression were needed [7]. So, researchers, WHO, people living with AIDS and NGO across the globe were actively focused for less expensive tools to measure TCD4+ cell in Low income countries. Manual TCD4+ methods such as Dynabeads (DynaL Biotech, Oslo, Norway) are less expensive alternatives to flow cytometry in resource-limited countries [9]. Previous study demonstrated the feasibility of the implementation of Dynabeads methods as an alternative technique to flow cytometry for TCD4+ lymphocytes counting in resource-limited settings [7]. However, this method requires fluorescent microscope which is expensive and not usually available in the majority of peripheral laboratories in these settings.

This study aimed (1) to evaluate several staining methods allowing an optimal counting of TCD4+ lymphocyte using Dynabeads method with light microscopy (DLM) and (2) to analyze its usefulness for TCD4+ lymphocyte enumeration in comparison to the Dynabeads method using fluorescence microscopy (DFM) and to the flow cytometry single platform method (FCM).

Materials and Methods

Study design and study population

This work was a cross-sectional comparative study conducted between January and September 2003 in the Microbiology and Immunology Lab of Centre Muraz, a biomedical and public health research institute based in Bobo-Dioulasso, Burkina Faso. The study population consisted of adults and children with an HIV-seropositive and HIV-seronegative status who regularly consulted the Centre Muraz for routine laboratory tests or for HIV cohort entry.

An effort has been made to include asymptomatic and pauci-symptomatic HIV-patients. Thus, the Dynabeads® technique using light microscopy could be tested in a wide range of values of CD4+ lymphocytes. In terms of immunoassays of TCD4+ lymphocytes, the inclusion of pediatric subjects is justified by the fact that children are known to have higher absolute lymphocytes than adults [10] (Diagbouga, Traoré, Ledru and Fumoux, unpublished data) and that it is also useful to test new techniques in populations with very high TCD4+ lymphocyte values.

Single platform flow cytometry technique

Blood samples were collected using EDTA-containing tubes and tested for TCD4+ count within 6 hours by flow cytometry using the BD FACScan (Becton Dickinson, San Jose, US), three-color mAb (TriTEST™ CD3FITC/CD4PE/CD45 PerCP) reagent and BD Trucount tubes according to standard procedures. Briefly, 50 μ l of freshly-obtained whole blood were stained with 20 μ l of anti-CD45-perCP, anti-CD3-FITC and anti-CD4-PE mAbs for 15 min at room temperature in the presence of a fixed number of fluorochrome-labeled, polystyrene reference beads. Red blood cells were lysed using a FACS Lysing Solution (BDIS) for 15 min at room temperature. Samples were then, analyzed using the FACScan and the Multiset and Attractor software (BDIS) for calculating absolute values of TCD4+ cells.

Internal quality control was established and systematized by the Centre Muraz microbiology and immunology lab. It consists essentially of the daily calibration and the optimization of the flow cytometer, the daily and weekly maintenance of the devices. The performance parameters of the cytometer are evaluated: analysis window, linearity, background noise, detector sensitivity. All these parameters were systematically analyzed and recorded in Quickcal (Flow Cytometry Standards Corp).

The Centre Muraz participated in the external quality control organized by the QASI Program an international program for quality assessment and standardization for immunological measures relevant to HIV/AIDS (Dr. John Fahey, Dr. Francis Mandy) and the UK National Quality Assessment Scheme for Leukocyte Immunophenotyping (Dr. David Barnett, Dr. JT Reilly, Mr. V. Granger).

Dynabeads technique (DynaL T4 QUANT kit)

The Dynabeads method (DynaL T4 QUANT kit, Dynal Biotech Oslo, Norway) using a staining with acridine orange and readings under a fluorescent microscope (AXIOLAB) for TCD4+ lymphocyte enumeration was done as described previously [7]. The technique used magnetic beads coated with anti-CD4 monoclonal antibodies (mAbs) to capture and isolate TCD4+ lymphocytes from whole blood. One hundred and twenty-five (125) μ l of freshly-obtained EDTA-anti-coagulated blood were added to 350 μ l of phosphate buffer saline (PBS). Twenty-five (25) μ l of suspended magnetic beads coated with anti-CD14 mAb were then added and the mixture incubated for 10 min at room temperature on a Dynal mechanical rotator in order to deplete blood from monocytes. Magnetic separation of monocytes was performed using a magnetic particle concentrator, as recommended by the manufacturer. One aliquot of 200 μ l was taken from the supernatant of monocyte depleted blood and dispensed into 200 μ l of PBS. Twenty-five microliters of beads coated with anti-CD4 mAb were then added prior to incubation at room temperature for 10 min on the rotator. The beads were separated using the magnetic particle concentrator and washed twice with PBS.

Table 2a Intern reproducibility at the threshold of 200 TCD4+ lymphocyte/ μ l.

	Mean	SD	CV%	Max	Min	Amplitude
AO	275	24	8.7	308	230	78
GIEMSA	320	23	7.1	350	280	70
TURCK	292	15	5.1	310	264	46
STMB	271	17	6.2	292	240	52
CMF	196	56	28.5	330	138	192

Table 2b Intern reproducibility at the threshold of 400 TCD4+ lymphocyte/ μ l.

	Mean	SD	CV%	Max	Min	Amplitude
AO	408	36	8.8	458	350	108
GIEMSA	499	54	10.8	576	426	150
TURCK	441	38	8.6	494	362	132
STMB	439	35	7.9	500	382	118
CMF	344	55	15.9	450	287	163

Table 2c Intern reproducibility at the threshold of 500 TCD4+ lymphocyte/ μ l.

	Mean	SD	CV%	Max	Min	Amplitude
AO	436	19	4.3	464	410	54
GIEMSA	544	40	7.3	604	476	128
TURCK	503	37	7.3	546	444	102
STMB	423	32	7.5	476	364	112
CM F	486	66	13.5	582	391	191

Table 2d Intern reproducibility at the threshold of 1000 TCD4+ lymphocyte/ μ l.

	Mean	SD	CV%	Max	Min	Amplitude
AO	1013	46	4.5	1090	948	142
GIEMSA	1055	85	8	1212	906	306
TURCK	1050	68	6.4	1172	906	266
STMB	913	50	5.4	980	864	116
CM F	986	104	10.5	1243	901	342

After addition of 50 μ l of lysing solution, cells were stained with 50 μ l of a solution of acridine orange and layered on a 10-band Malassez Cell where they were integrally enumerated using a fluorescent microscopy. Results were expressed as a number of positive cells per microliter of whole blood.

Selection of dyes allowing TCD4+ lymphocytes enumeration under light microscopy

After a bibliographic search on the potential dyes of the lymphocyte nuclei, we have compiled a list of eleven different methods of staining. The procedure for preparing the dyes is shown in the Annex. We then tested the different coloring methods on a number of 15 patients. The various criteria of this first essential qualitative assessment of the ease of nucleus counting under the light microscope included: the appearance of the bottom of the Malassez cell, the appearance of the nucleus, the contrast, etc. An advanced research on three (3) patients among the 15 subjects allowed us to assign one value out of 10 to each of these dyes. Five technicians gave an appreciation (in a completely blind and independent manner of the appreciation of his colleagues). The averages of the five (5) values are presented in **Table 1**. These various researches led us to retain for the rest of the work the three (3) best dyes which were the Sternheimer-Malbin solution (STMB), the Turck1 solution, and the Giemsa solution.

For the use of Dynabeads method with light microscopy, the same procedure was used and differences appeared after the beads were separated using the magnetic particle concentrator and washed twice with PBS to isolate TCD4+ cells. Then, the procedure was done in respect with the type of dyes as described in the Annex.

Statistical analysis

Statistical analyses were performed using the SAS-PC software (version 8.2, SAS Institute, Cary, North Carolina, USA). The independent variable analyzed was the absolute TCD4+ count. Flow cytometric testing was compared with each Dynabeads method used. Paired data were compared by linear regression, including slopes, intercepts, and Pearson correlation coefficients. Analysis of agreement between the 2 methods was done by the Bland and Altman method, which compares the difference between paired measurements against the mean of the 2 measurements. P-values were the results of two-sided tests and considered as significant if below 5%. Difference between the two techniques was computed on each sample as the subtraction of the FCM result to the Dynabeads result. The correlation coefficient between the two techniques was calculated and a model of regression then applied to the data.

To assess the accuracy of the Dynal technique using light microscopy, 4 fresh blood samples (200, 400, 500 and 1000 TCD4+/ μ l in FCM) have been analyzed ten times for each dye against the fluorescence microscopy used with acridine orange.

Positive predictive value (PPV) and negative predictive value (NPV) were computed at the level of 200 and 350 TCD4+

obtained by flow cytometry for the Dynabeads staining using acridine orange, Sternheimer-Malbin, Turck1 and Giemsa.

Table 1 Qualitative evaluation of the dyes used with light microscopy.

Dyes with type of microscopy	Storage conditions	Remarks	Background appearance of Mallassez slide	Appearance of TCD4+ nuclei	Contrast	Ranks/10
Acridine orange fluorescence microscopy	+4°C	Very comfortable to read and very easy to read	Green	fluorescent Yellow green	Excellent	10/10
Sternheimer-Malbin light microscopy	+4°C	Less tiring for eyes	Pale violet	Dark purple	Good	7/10
Turck1 (purple gentian) light microscopy	+25°C	Good contrast but light is intense	Yellow- pale green	Purple	Good	7/10
Turck2 (methylene blue) light microscopy	+25°C	Good contrast but light is intense	Yellow green	Light yellow	Good +/-	6/10
/Coulter reagent light microscopy	+4°C	Contrast is not excellent	Brown	Dark purple	Good+/-	5/10
Giemsa Light microscopy	+25°C	Good contrast	Pale blue	Purple	Good	7/10
Trypan blue light microscopy	+25°C	Contrast is not excellent	Yellow	Blue cells enveloped by beads	-	2/10
PBS buffer light microscopy	+4°C	Good contrast	Yellow-green	Yellow-green	-	5/10

Ethical approval

The study protocol was approved by the Centre Muraz Institutional Review Committee

Patient's informed consent was obtained prior to inclusion in the study

Results

Population characteristics

A population of 305 participants was recruited. They were 139 HIV-seropositive and 166 HIV-seronegative individuals and consisted of 250 adults (99 HIV+ and 151 HIV-) and 55 children (40 HIV+ and 15 HIV-). Age of participants was between 20 and 63 years for adults and 1.5 to 17 years for children.

Qualitative assessment of the dyes used with light microscopy and their classification

The procedure for the preparation of dyes is presented in the Annex. The average of the 5 values gave by the technicians are presented in **Table 1**. Score of readings with acridine orange used with fluorescent microscopy was considered as the reference of the readings. It was scored 10/10. The best's score were obtained with Sternheimer-Malbin (7/10), Turck1 (7/10) and Giemsa (7/10).

The nuclei colored with the Sternheimer-Malbin solution appeared dark purple in microscopy under white light. The nuclei colored with Turck1 solution appeared purple under

white light and nuclei colored by Giemsa solution appeared purple. The comfort of reading is tiny room compared to a staining with acridine orange and, consequently, a fall of sensitivity and reproducibility is to be feared with a reading in microscopy in white light. Turck1 and Sternheimer-Malbin dyes were preferred by the technicians.

After this qualitative evaluation, the three best's dyes were used for the evaluation of Dynal T4 Quant using light microscopy in comparison with flow cytometry and Dynal T4 Quant using fluorescent microscopy.

Reproducibility of the Dynabeads method using light microscopy (DLM) at the threshold of 200, 400, 500 and 1000 TCD4+ lymphocyte/ μ l

Four fresh blood samples with approximately 200, 400, 500 and 1000 TCD4+ lymphocytes per μ l in flow cytometry were analyzed 10 times with Dynabeads techniques using light microscopy according to the three dyes: Giemsa, Turck1, Sternheimer-Malbin (STMB), and with Dynabeads technique using fluorescence microscopy and acridine orange (AO) and the single flow cytometry platform. The mean of TCD4+, the standard deviation (SD), the coefficient of variation (CV), and amplitude of each technique at different levels of TCD4+ were shown in **Tables 2a to 2d**. At all levels of TCD4+ lymphocyte, FCM was the technique showing the greatest discrepancies. The reproducibility of Dynabeads® techniques was improved. The Dynabeads method using acridine orange was the technique with the lowest standard deviations except at level

200. Giemsa gave overall values higher than other Dynabeads® techniques. At 200 TCD4+ lymphocyte threshold, cytometry gave lower values and was the technique with the greatest deviations. At 400 TCD4+ lymphocyte threshold, cytometry gave lower values, however, closer to other techniques than for level of 200. FCM was the technique with the greatest deviations at this level. At 500 TCD4+ lymphocyte threshold, cytometry gave values close to other techniques. FCM was the technique with the widest gaps at this level.

At 1000 TCD4+ lymphocyte threshold, examination of the graph shown that cytometry gave values close to other techniques. FCM was the technique with the widest gaps at this level.

Table 3 Correlation coefficient between Dynabeads methods using light or fluorescent microscopy and the flow cytometry for TCD4+ lymphocytes counting (55 children and 119 adults).

Children and adults	Acridine Orange	Giemsa	Turck 1	STMB	FCM
Acridine	1				
Giemsa	0.98	1			
Turck1	0.99	0.98	1		
STMB	0.99	0.98	0.99	1	
FCM	0.975	0.969	0.972	0.973	1
Children only	Acridine Orange	Giemsa	Turck 1	STMB	FCM
Acridine	1				
Giemsa	0.97	1			
Turck1	0.98	0.97	1		
STMB	0.98	0.95	0.98	1	
FCM	0.96	0.94	0.95	0.95	1

Overall correlation between Dynabeads method using light microscopy and flow cytometry single platform method

The correlations between the different techniques were studied for the absolute values of the TCD4+ lymphocyte obtained from 55 children (40 HIV+ and 15 HIV-) and 119 adults (99 HIV+ and 21 HIV-) (**Table 3**). The correlation coefficient was better between FCM and DFr. The correlation coefficient was better for adults than for children. However, it was acceptable in all cases. The lowest correlation coefficient was obtained with Giemsa. All the correlation coefficients were higher. The overall Pearson correlation coefficient obtained between Dynabeads methods versus FACScan were 0.975, 0.969, 0.972, and 0.973 respectively for acridine orange (AO), Giemsa, Turck1 and Sternheimer-Malbin (STMB). Very best correlation with the flow cytometry was obtained with the Dynabeads technique using acridine orange with fluorescent microscopy. However, correlation obtained with the other dyes used with light microscopy was also good; the weakest correlation was obtained with Giemsa dye.

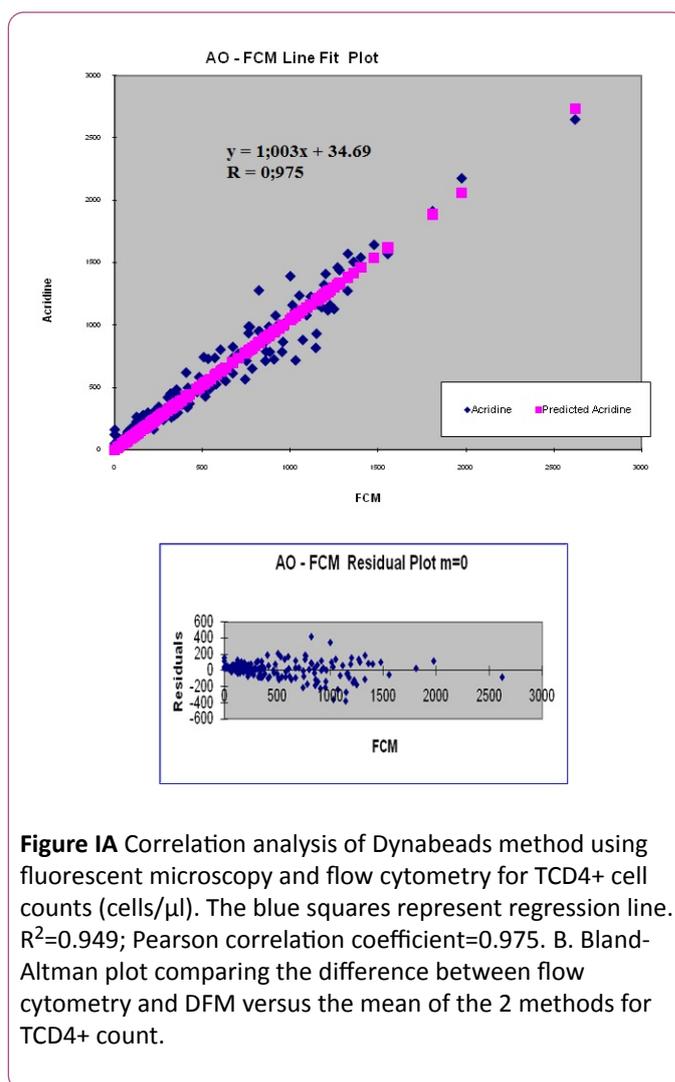
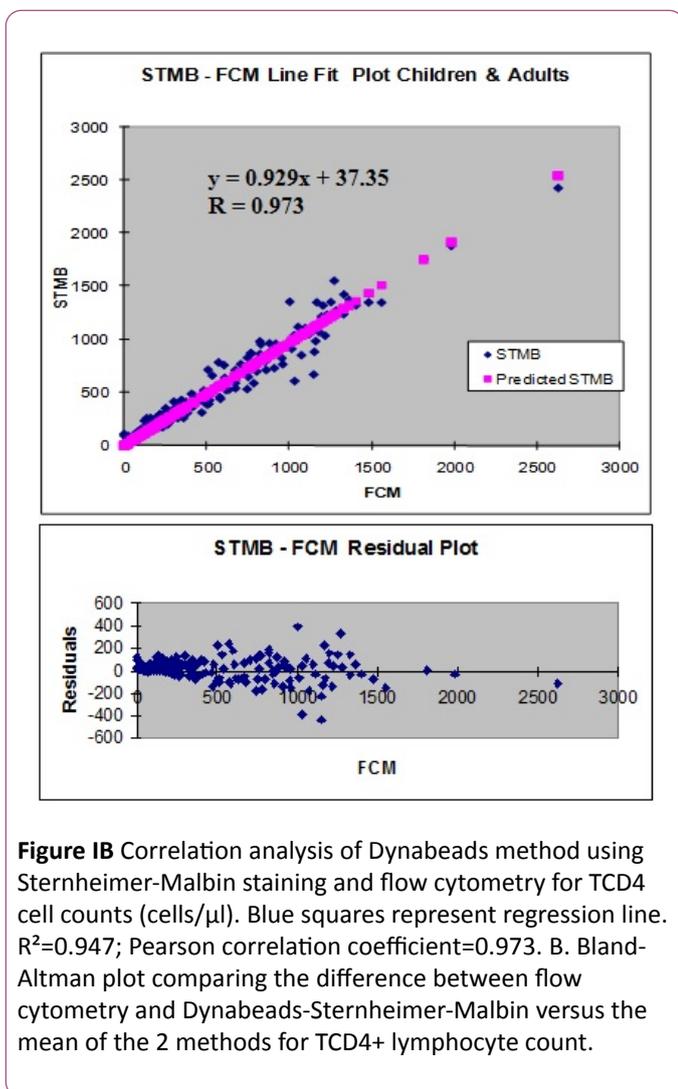


Figure 1A Correlation analysis of Dynabeads method using fluorescent microscopy and flow cytometry for TCD4+ cell counts (cells/ μ l). The blue squares represent regression line. $R^2=0.949$; Pearson correlation coefficient=0.975. B. Bland-Altman plot comparing the difference between flow cytometry and DFM versus the mean of the 2 methods for TCD4+ count.

Figure 1A shows the line of regression between Dynabeads CD4 using acridine orange and FACScan where most of the values lay close to the regression line showing that there was a good agreement between Dynabeads using acridine orange and FCM. The correlation between TCD4+ counts assessed by Dynabeads using acridine orange and flow cytometry was 0.975 ($y=1.003x + 34.69$; $R^2=0.949$). **Figure 1B** shows the line of regression between Dynabeads using Sternheimer-Malbin and FACScan where most of the values lay close to the regression line showing that there was a good agreement between Dynabeads using Sternheimer-Malbin and FACScan methods. The correlation between TCD4+ counts assessed by Dynabeads using Sternheimer-Malbin and flow cytometry was 0.97 ($y=0.929x + 37.35$; $R^2=0.947$). **Figure 1C** shows the line of regression between Dynabeads using Turck1 and FACScan where most of the values lay close to the regression line showing that there was a good agreement between Dynabeads using Turck1 and FACScan methods. The correlation between TCD4+ counts assessed by Dynabeads using Turck1 and flow cytometry was 0.972 ($y=0.929x + 37.35$; $R^2=0.947$). **Figure 1D** shows the line of regression between Dynabeads CD4 using Giemsa and FACScan where most of the values lay close to the regression line showing that there was a good agreement between Dynabeads using Giemsa and

FACScan methods. The correlation between TCD4+ counts assessed by Dynabeads using Giemsa and flow cytometry was 0.969 ($y=1.038x + 15.32$; $R^2=0.938$).



Predictive values

Acridine orange, Giemsa, Turck1 and Sternheimer-Malbin were evaluated for their ability to correctly segregate absolute TCD4+ at the 200 and 350 TCD4+ lymphocyte/ μ l thresholds.

The positive predictive values for a TCD4+ lymphocyte count higher than 200 cells/ μ l by FCM for 174 participants were respectively 98.4%, 96.8% for acridine orange and Giemsa, 96% for Turck1 and Sternheimer-Malbin. The negative predictive values for a TCD4+ count less than 200 cells/ μ l were 73.47%, 75.51%, 91.84%, and 87.75% respectively for acridine orange, Giemsa, Turck1 and Sternheimer-Malbin. The global discrepancies were 8.62%, 9.19%, 5.17% and 6.32% respectively for acridine orange, Giemsa, Turck1 and Sternheimer-Malbin (**Table 4**).

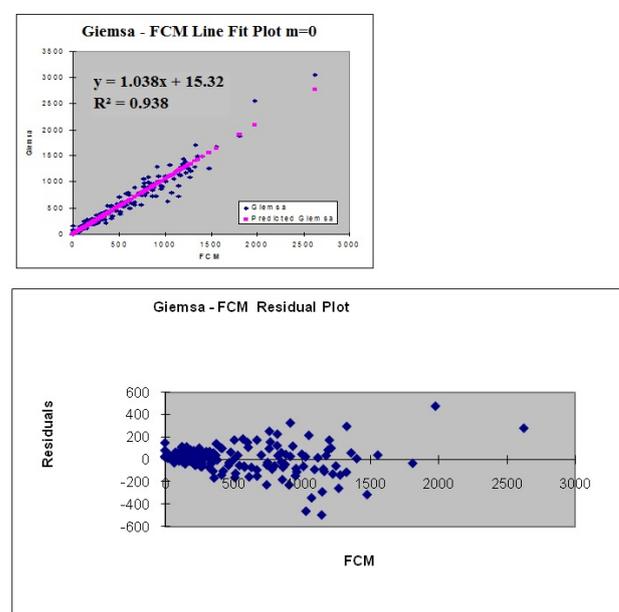
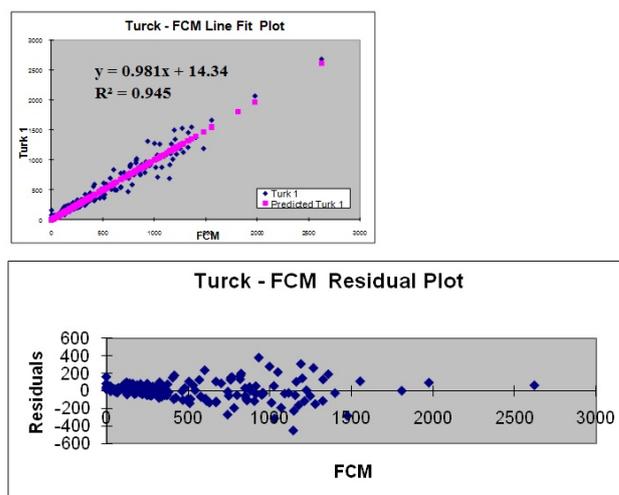


Table 4 Segregation of individuals with use of TCD4+ lymphocytes at 200 TCD4+/ μ l level: FACSCan versus Dynabeads using study dyes.

FCM 200	FCM<200 (VPN)	FCM>200 (VPP)
AO<200	36	2
AO>200	13	123
Giem.<200	37	4
Giem.>200	12	121
Turck1<200	45	5
Turck1>200	4	120
STMB<200	43	5
STMB>200	6	120
FCM<200	49	0
FCM>200	0	125

Data are number of individuals. P<0.001 (Chi-Square test). Positive predictive values for a TCD4+/ μ l \geq 200 were 98.4%, 96.8% for acridine orange and Giemsa, 96% for Turck1 and Sternheimer-Malbin. Negative predictive values for TCD4+ lymphocyte/ μ l <200 were 73.47%, 75.51%, 91.84%, and 87.75% respectively for acridine orange, Giemsa, Turck1 and Sternheimer-Malbin

The positive predictive values for a TCD4+ lymphocyte count higher than 350 cells/ μ l by FCM for 174 participants were 97.47% for all the dyes.

Table 5 Segregation of individuals with use of TCD4+ lymphocytes at 350 TCD4+/ μ l level: FACSCan versus Dynabeads using study dyes.

FCM 350	FCM<350 (NPV)	FCM>350 (PPV)
AO<350	90	2
AO>350	5	77
Giemsa<350	86	2
Giemsa>350	9	77
Turck1<350	94	2
Turck1>350	1	77
STMB<350	91	2
STMB>350	4	77
FCM<350	95	0
FCM>350	0	79

Data are number of individuals. P<0.001 (Chi-Square test). Positive predictive values for a TCD4+ lymphocyte/ μ l \geq 350 were 97.47% for all the dyes used in the study. Negative predictive values for TCD4+ lymphocyte/ μ l <350 were 94.74%, 90.53%, 98.95% et 95.79% respectively for acridine orange, Giemsa, Turck1 and Sternheimer-Malbin

The negative predictive values for a TCD4+ count less than 350 cells/ μ l were 94.74%, 90.53%, 98.95% and 95.79% respectively for acridine orange, Giemsa, Turck1 and Sternheimer-Malbin. The global discrepancies were respectively 4.02%, 6.32%, 1.72% and 3.45% respectively for

acridine orange, Giemsa, Turck1 and Sternheimer-Malbin (Table 5).

Discussion

Due to the increased demand of TCD4+ lymphocyte enumeration in resource-limited settings, this study intended to allow the large decentralization of Dynabeads method in central and peripheral laboratories which have the possibility to possess light microscopy for routine biomedical analyzes. Qualitative evaluation of dyes led us to select the three bests which could be used efficaciously with light microscopy. The specificity of these dyes is that they are used in medical laboratory for routine diagnosis. Therefore, the light microscopy is the main tool in the labs at the district levels and the small laboratories. Giemsa solution is used with May-Grünwald in routine for detection of malaria parasites on stained thick and thin blood films in research settings [11] for hematology parameters analyses, diagnosis of malignant cells [12] and for male fertility exam by the detection of spermatogenic [13]. Sternheimer-Malbin protocol is also used in hematology and for the research of leucocytes in urine [14]. Turck's solution is also used in hematology for white blood cells differentiation [15]. Because these dyes are usually used in routine laboratory activities, they will be useful for TCD4+ enumeration of HIV patients in rural areas and peripheral districts with limited logistics. Acridine orange is used for vital staining in hematology, apoptosis, bone marrow staining [16,17] and in parasitological analyses [18]. Dynabeads method using acridine orange with fluorescent microscopy was evaluated in many settings with good correlation compared to the flow cytometry TCD4+ results [7,19-21]. In Japan, modified protocol of the Dynabeads method was assessed by the used of reducing volume of anti-CD14 and anti-CD4 and a significant correlation of 0.91 with flow cytometry was obtained [21]. In this study, the positive and negative predictive values for a TCD4 count less than 350 cells/ μ l were good, 97% and 83%. The negative predictive values for a TCD4+ less than 350 cells/ μ l were also good: 94.74%, 90.53%, 98.95% and 95.79% respectively for acridine orange, Giemsa, Turck1 and Sternheimer-Malbin in our study. Sternheimer-Malbin solution with Dynabeads technique and counted under light microscopy was used in Uganda and the correlation coefficient was good (0.85) [22]. In our study, the use of Sternheimer-Malbin solution shows an excellent correlation coefficient of 0.973, while correlations coefficients with Turck1 and Giemsa solutions were also excellent. In our study, at all levels of TCD4+ count, FCM is the technique that presents the greatest standard coefficient of variation and amplitude. Coefficients of variation obtained with the stains were better than the coefficient of variation obtained with flow cytometry technique. Reproducibility was best with Dynabeads techniques than flow cytometry. The Dynabeads method using acridine orange is the technique with the lowest standard deviation, with the exception of the 200 TCD4+ level. For 350 TCD4+/ μ l or 200 TCD4+/ μ l thresholds, the concordance between FCM and the numeration with the Turck1 solution is 98% and 95%, respectively. The concordance between FCM and the numeration with the STMB is 97% and

93%, respectively. The use of Giemsa solution generates lower correlations and higher standard deviations.

The main limitations of Dynabeads technique include its requirements several steps, its chronophage nature and its precision that could be rely on operator expertise. The technique could seem complex for a new user but after long time of using the technique become simple and easy to use. Dynabeads technique requires refrigeration of reagents, a microscope with a 40X objective, a hemocytometer, calibrated pipettes, test tubes, and a manual counter [23].

The use of the selected dyes with light microscopy could substantially reduce the price of TCD4+ lymphocyte enumeration in resource limited settings. With the development of alternative flow cytometry and point of care CD4 technologies by the firms and the efforts of international organisms, non-governmental organizations to decentralize monitoring tools for HIV care in resource limited settings Dynabeads techniques using light microscopy could be in the light of these policies to be implemented in rural areas and peripheral districts. In addition, Dynabeads technology would be implemented in the central laboratories also to face up to the reagents out of stock, breakdown of flow cytometers for the continuing care of HIV patients. A study was demonstrated the feasibility of automation of Dynal T4 Quant in hematology automate as a Point of Care CD4 with the delivering in the same time of hematology parameters and TCD4+ counting [4]. To improve on throughput using the manual Dynal T4 Quant method, the Sysmex pochH-100i hematological analyzer was used to count stained nuclei and showed 93% concordance with FCM [4]. The capacity to use the Dynal T4 Quant with hematology automate by the using K-Xpert software was successfully validated [4]. In April 2002, WHO recommended that when TCD4 cell count is not available or is not affordable to be obtained for affected individuals, a total lymphocyte count of less than 1000-1200 lymphocytes/ μ l in individuals with stage 2 or stage 3 diseases to be used as an indication to initiate antiretroviral therapy [24]. To the best of our knowledge, studies comparing the utility of absolute lymphocyte counts (ALC) as a surrogate for CD4 count in monitoring HIV infected individuals have shown contradictory results. Indeed, many published studies showed low sensitivity of absolute lymphocyte count less than 1200/ μ l for predicting CD4 cell count less than 200 cells/ μ l [25-27]. Conversely, studies from Karanth et al. and Obirikorang et al. [28,29] shown higher sensitivity and specificity for total lymphocyte count cut-off 1200 cells/ μ l to predict CD4 cell count less than 200/ μ l. The differences could be due to different ethnic, racial, epidemiological and socioeconomic factors.

Conclusion

Dynal T4 Quant technique is an affordable and accurate method for TCD4+ counting and it is an effective alternative TCD4+ method for resource-limited settings, despite the development of many news alternatives methods for TCD4+ screening. Following this study, it has been demonstrated that reliable TCD4+ numeration can be obtained with the Dynal T4 Quant Kit, in light microscopy with the Sternheimer-Malbin

Turck1 and Giemsa solutions. These results could contribute to further reduce the cost of TCD4+ lymphocytes counting and make it more widely available in peripheral laboratories and even in central laboratories that face problems with maintenance and stock-out of reagents for flow cytometers.

Authors Contributions

SD and LC designed the study, HH, AO recruited the study population, SD, EB, AK, DZ, AO performed the experiments, SD, LC and DZ analyzed the data SD, LC, DZ, EB wrote the manuscript which was approved by all the other co-authors.

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