Affordable CD4 Enumeration for HIV Staging in Resource-constrained Settings

Xiao Li
The research described in this thesis was carried out at the Biophysical Engineering Group, Faculty of Science and Technology, University of Twente, Enschede, The Netherlands.

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By Xiao Li

Ph.D. Thesis, with references; with summary in English, Dutch and Chinese
University of Twente, Enschede, The Netherlands, January 2008

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Cover designed by Xiao Li.
The Red Ribbon is the international symbol of HIV and AIDS awareness. It stands for: Care and Concern, Hope, and Support. Back cover: Map of Africa coloured according to the adult HIV/AIDS prevalence rate (adapted from UNAIDS).

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Affordable CD4 Enumeration for HIV Staging in Resource-constrained Settings

DISSERTATION

to obtain
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on the authority of the rector magnificus,
prof. dr. W.H.M. Zijm,
on account of the decision of the graduation committee,
to be publicly defended
on Friday, the 11th January 2008 at 13.15 hrs.

by

Xiao Li

born on the 5th March 1976
in Huangshan, P. R. China
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To Zheng and My Parents...
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1.1 Human Blood

Human blood is a suspension of blood cells in plasma. 55% of the blood in volume is plasma. It is a straw-colored fluid, which carries dissolved substances including nutrients, waste products, ions and proteins. The rest is a mixture of red blood cells (RBCs or erythrocytes), white blood cells (WBCs or leukocytes), and platelets (or thrombocytes). Human blood works together with the lymphatic system to provide a highly efficient defense system (1).

RBCs (>99% of all blood cells; 4 - 6 million/µl of blood) are biconcave, disk-like cells, rich in hemoglobin molecules (~280 million/cell), and lack a nucleus; they carry oxygen to cells at different locations of the human body and transport carbon dioxide back to lung. Platelets (~250,000/µl of blood) are tiny membrane enclosed packets of cytoplasm; they play an important role in blood clotting. WBCs (5,000 - 9,000/µl of blood) have nuclei but do not have hemoglobin; they perform various vital roles in the immune system (1, 2).

WBCs are classified into two categories: granular WBCs and agranular WBCs. Granular WBCs contain numerous granules in the cytoplasm, and their nuclei are lobed. They are produced in the bone marrow and comprise 3 types: 1. Neutrophils (50 - 70% of WBCs) are the most common WBCs in the blood stream. They are highly mobile and aggressive, phagocytizing pathogens and cellular debris. They are the first WBCs that appear at the site of an injury. 2. Eosinophils (1 - 4% of WBCs) help break down blood clots and kill parasites. 3. Basophils (<1% of WBCs) synthesize and store histamine (a substance released during inflammation and allergic response) and heparin (an anticoagulant) (2).
Agranular (or non-granular) WBCs have few or no granules in the cytoplasm and have a large spherical nucleus. They are produced in lymph tissue, and comprise two types. 1. Monocytes (2 - 8% of WBCs) are large phagocytes (typically as macrophages in tissues of the liver, spleen, lungs, and lymph nodes), and are also antigen-presenting cells. 2. Lymphocytes (25 - 40% of WBCs) play an important role in the immune response.

There are three sub-types of lymphocytes. a. Natural killer cells (NK cells) kill cancerous and virus-infected cells with a corrosive enzyme. b. B lymphocytes (CD19+ B lymphocytes) turn into antibody producing plasma cells when activated. Each B lymphocyte is specialized for a specific antibody. c. T lymphocytes (CD3+ T lymphocytes) comprise two varieties: Cytotoxic T cells (CD8+ T lymphocytes) and Helper T cells (CD4+ T lymphocytes). Cytotoxic T cells kill pathogens identified by the immune system, for instance, the pathogens tagged by antibodies. Helper T cells play a vital role in maintaining the integrity of the human immune system, stimulating the differentiation of B lymphocytes and cytotoxic T cells, and maximizing the bactericidal activity of phagocytes such as macrophages (2). (CD (cluster of differentiation) numbers are used to identify cell surface antigens (Ags) that can be distinguished by monoclonal antibodies (MAbs).) In T cells, there is a difference between naïve T cells, activated T cells and memory T cells. Naïve T cells are mature, but have not yet encountered a cognate antigen in the periphery. After encountering an antigen, naïve T cells become activated and begin to proliferate into different clones. Some will differentiate into activated T cells that will perform the specific function of that cell (e.g. producing cytokines in the case of helper T cells or invoking cell killing in the case of cytotoxic T cells). Some will differentiate into memory T cells that will survive in an inactive state in the host for a long period of time until they re-encounter the same antigen and reactivate. It is essential for an immune system to have adequate numbers of naïve T cells to be able to adapt to new pathogens experienced in life (2).

The morphology of the above mentioned types of WBCs is shown in Figure 1.
WBCs provide the body with two kinds of immune responses: innate immunity and specific immunity (adaptive immunity). Neutrophils and NK cells provide the innate immunity by attacking any suspicious antigen. T and B lymphocytes, together with antibodies (Abs), provide the specific immunity by recognizing and responding to specific antigens and giving a bigger, faster and more effective response than the innate immunity does (2).

1.2 HIV Infection and CD4⁺ T Lymphocytes Depletion

Human immunodeficiency virus (HIV) is a retrovirus that causes acquired immunodeficiency syndrome (AIDS), in which the immune system turns to fail, leading to life-threatening opportunistic infections. There are two species of HIV: HIV-1 and HIV-2. HIV-1 is easily transmitted and virulent, which causes the majority of HIV infections globally. HIV-2 is less transmittable and largely confined in West Africa (2, 3). There is no cure to HIV infection, but drugs that interfere with viral replication can slow down the development of HIV disease. The “highly active antiretroviral therapy” (HAART) can reduce the viral load in the peripheral blood to nearly undetectable levels. Patients infected with HIV who are treated by HAART are now living much longer and are healthier than before (2).

HIV infects primarily a specific population of vital cells in the human immune system, i.e. the helper T cells (specifically CD4⁺ T lymphocytes), and kills them. The entry of HIV into CD4⁺ T lymphocytes is mediated through the interaction of the virion envelope glycoproteins (gp120) with the CD4 molecule on the target cells and also with chemokine co-receptors CXCR4 or CCR5 (2, 4). Similar to any other infectious agent,
HIV presents its proteins to the immune system, which may develop antibodies against it. However, the antibody production is hampered by the fact that HIV mutates rapidly (2).

FIG. 2. A generalized graph of the relationship between the CD4⁺ T lymphocytes count and the HIV viral load along the average course of untreated HIV infection.

HIV infection is associated with a progressive decrease of the CD4⁺ T lymphocytes count and an increase in viral load, as shown in Figure 2. Immediately following the exposure of HIV to a patient, the primary or acute infection stage starts, in which the HIV viral load in the peripheral blood increases rapidly to a level of several million/ml. This response is accompanied by a remarkable drop of the circulating CD4⁺ T lymphocytes number. During this period (usually 2 - 4 weeks post-exposure), most patients (80 - 90%) develop an influenza or mononucleosis-like illness called acute HIV infection. Then, an immune response to HIV is activated, followed with a decrease in detectable viral load and a slight increase of CD4 count. A period of clinical latency follows, during which CD4 counts continue to decrease and the viral load continue to increase slightly. Clinical latency can vary between two weeks and 20 years. Opportunistic infections and other symptoms start at around 500 cells/μl, and become
more frequent as the CD4 count falls; at this moment, the disease enters the sympotmatic phase. When CD4 counts fall below 200 cells/μl, and the viral load increases dramatically, the cell-mediated immunity is lost and infections with a variety of opportunistic microbes appear. The patient is said to have AIDS. If untreated, eventually most HIV-infected people will develop AIDS and die (2, 5).

HIV kills CD4+ T lymphocytes by three main mechanisms: 1. Directly killing the infected cells by cytolysis or by membrane fusion between cells to form a giant multinucleated cell (syncytium) that has a short lifespan; 2. Killing infected cells by CD8 cytotoxic lymphocytes that recognize infected cells; 3. In-directly killing infected or uninfected cells by inducing the cells to "commit suicide" (apoptosis; programmed cell death) (2, 5 - 7).

CD4+ T lymphocytes in circulating blood constitute only about 1 - 2% of the total of these cells that are present in the entire immune system, while the majority of the lymphocytes are sequestered in lymphoid tissues (2, 5 - 7). Gut-associated lymphoid tissue (GALT) harbors the majority of T lymphocytes in the body and is an important target for HIV-1. Recent literatures report a massive loss of memory CD4+ T lymphocytes in mucosal tissues, the major reservoir for memory CD4+ T cells in adults, particularly in GALT, within the first three weeks of infection of rhesus macaques by simian immunodeficiency virus (SIV) and of human by HIV (8 - 12). This phenomenon is in contrast to the gradual decline of CD4+ T lymphocytes count in the peripheral blood. This initial strike to the immune system is found to be the distinguishing characteristic of HIV-1 pathogenesis. Its extent determines the overall course of the infection and progression to AIDS (10, 11). It was reported that 30 - 60% of the memory CD4+ T lymphocytes throughout the body are infected with SIV at the infection peak and killed by the 14th day after infection. This high infection rate suggested that the virus induced cytolysis or removal by cytotoxic T cells is probably the main cause for the initial depletion of infected CD4+ T lymphocytes (10). Surprisingly, the cells infected with HIV-1 within the first two weeks of the infection are not necessarily in an activated state but rather expressed as a "resting"-like phenotype. Actually only < 1% of the infected cells actively produces new viral particles during the chronic phase of infection (13). The
CD4+ T lymphocytes infected with HIV-1 in blood during the chronic stage is only about 0.01 - 0.10% (13, 14). This low infection frequency cannot cause the large elimination of CD4+ T lymphocytes. A major mechanism for the depletion of both infected and especially uninfected CD4+ T lymphocytes is apoptosis, which can be induced by HIV through different pathways: apoptosis in both infected and uninfected cells by a Fas-FasL-mediated mechanism during activation-induced cell death (AICD) (5, 15); apoptosis in uninfected cells stimulated by HIV proteins (Tat gpl20, Nef, Vpu) released from infected cell. Recently it was reported that IFN-α induces only CD4+ T lymphocytes to express TNF-related apoptosis-inducing ligand (TRAIL). Concurrently, HIV-1 viron up-regulates the expression of the death receptor DR5 on CD4+ T lymphocytes. The interaction of TRAIL with DR5 induces the selective apoptosis of only CD4+ T lymphocytes but not CD8+ T lymphocytes (16, 17).

In conclusion, CD4+ T lymphocytes are depleted by several concurrent mechanisms depending on the particular tissue environment, and the predominant mechanisms in the acute phase differ from those in the chronic phase.

The near-complete elimination of memory CD4+ T cells in mucosal lymphoid tissues during the initial acute infection stage is partly compensated by the continuous differentiation of naïve CD4+ T cells into memory CD4+ T cells. However, these recruited memory CD4+ T cells are rapidly killed. This replenishment process eventually leads to the exhaustion of the naïve CD4+ T cell pool and the development of disease to the final stage (18). The early ART immediately after infection could be highly beneficial as it limits the damage to the memory CD4+ T cells and efficiently maintains HIV-specific CD4+ T cell response.

1.3 HIV/AIDS Situation and Immunological Monitoring of CD4 Count

Since the official date for the beginning of the AIDS epidemic in 1981 in Los Angeles (19), HIV infection has become a global pandemic major health emergency. This epidemic has formed a serious, and in many countries devastating, crisis. It is estimated that in 2006, 39.5 (34.1 - 47.1) million human beings were living with HIV, 4.3 (3.6 - 6.6) million were newly infected, and 2.9 (2.5 - 3.5) millions died in this year.
Among the 39.5 million people infected with HIV, approximately 95% were living in developing countries (20).

In recent years, due to the introduction of the generic ART, the price of ART per person per year has dramatically fallen from US$ 10,000 to US$ 300. By the end of 2005, about 1.3 million persons in middle- and low-income countries had received this treatment (20). Although the inexpensive treatment becomes more and more available for patients in the resource-constrained countries, the routine tests for HIV management in the west, e.g. CD4+ T lymphocytes count and plasma HIV load, are out of reach elsewhere. Since viral load tests (~ US$ 100 per test) are far too expensive for resource-constrained countries, the World Health Organization (WHO) recommends CD4+ T lymphocytes enumeration for HIV management there.

CD4+ T lymphocytes are coordinators of the immune response, but unfortunately they are the primary targets of the HIV. The number of CD4+ T lymphocytes, being complementary to HIV plasma viral load (as shown in Figure 2), provides information about how far the HIV infection disease has been developed (21, 22). It is now universally accepted that by definition AIDS is reached when the CD4+ T lymphocytes count falls to below 200/μl or 14% of total lymphocytes (23). The CD4+ T lymphocytes count in the peripheral blood is the most important parameter to determine the disease stage and progression, to assist in decisions regarding when to start or change ART, and to assess treatment effect (24 - 26).

The WHO proposed clinical criteria to stage HIV infection in association with low-cost laboratory tests. According to clinical manifestation and performance, patients are classified into 4 clinical stages. The stage I is classified for asymptomatic patients, and the stages II-IV are for patients with mild, advanced and severe HIV-associated clinical disease, respectively (27). The National AIDS Control Organization (NACO) has published a guideline for initiation of ART in adults and adolescents with WHO stage IV disease irrespective of CD4 count, with WHO stage III disease and CD4 count < 350/μl, and with WHO stage I and II disease and CD4 count < 200/μl (28). Since the CD4 count may vary between different ethnicities, it is important to establish the reference ranges of CD4 count for the different target populations (29 - 32).
For children patients, the reference range of CD4 and CD8 values as percentage of total lymphocytes, the absolute numbers of CD4⁺ T and CD8⁺ T lymphocytes, and CD4/CD8 ratio are different from those of adults (33 - 35). The criteria for HIV staging of children are different from that for adults, as shown in Table 1 (36 - 37). In children the CD4 percentage shows less age-related variability than the absolute CD4 count. Therefore, CD4 percentage or CD4/CD8 ratio is more informative and used for pediatric HIV monitoring. The WHO recommends initiation of ART for HIV positive infants and children with WHO Pediatric Stage III and IV disease irrespective of CD4 count, and for those with WHO Pediatric Stage I and II disease with CD4 counts or %CD4 at the aged specific cut-off levels (≤ 11 months: CD4 < 1500/μl or %CD4 < 25%; 12 - 35 months: CD4 < 750/μl or %CD4 < 20%; 36 - 59 months: CD4 < 350/μl or %CD4 < 15%; ≥ 5 years: CD4 < 200/μl or %CD4 < 15%) (38).

**Table 1.** Immunological categories based on age-specific CD4⁺ T lymphocytes counts and percentage of CD4⁺ T lymphocytes in total lymphocytes.

<table>
<thead>
<tr>
<th>Immunological classification</th>
<th>Children &lt; 13 years</th>
<th>Adolescents &gt;13 years and adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>No suppression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥ 1500/μl</td>
<td>≥ 1000/μl</td>
<td>≥ 500/μl</td>
</tr>
<tr>
<td>≥ 25%</td>
<td>≥ 25%</td>
<td>≥ 25%</td>
</tr>
<tr>
<td>Moderate suppression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>750 - 1499/μl</td>
<td>500 - 999/μl</td>
<td>200 - 499/μl</td>
</tr>
<tr>
<td>15 - 24%</td>
<td>15 - 24%</td>
<td>15 - 24%</td>
</tr>
<tr>
<td>Severe suppression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 750/μl</td>
<td>&lt; 500/μl</td>
<td>&lt; 200/μl</td>
</tr>
<tr>
<td>&lt; 15%</td>
<td>&lt; 15%</td>
<td>&lt; 14%</td>
</tr>
</tbody>
</table>

1.4 CD4 and CD8 Enumeration Technologies

A comparison of CD4 enumeration methods is summarized in Table 2.

**FCM methods for CD4 enumeration**

**Conventional FCM**

Flow cytometry (FCM) is the widely accepted gold standard method for CD4 enumeration due to its accuracy, precision and reproducibility. CD4 enumeration can be accomplished by dual platform (DP) or single platform (SP) FCM methods. In the DP...
FCM methods, absolute CD4 count is calculated by multiplying the percentage of CD4+ T lymphocytes in the total lymphocytes obtained by FCM with the absolute lymphocytes count obtained by an automatic hematology analyzer (23). Accuracy of the DP FCM methods is largely dependent on the definition of the lymphocytes counted by a hematology analyzer. The SP FCM methods use calibration beads (39, 40) or employ a volumetric method (41, 42) to achieve an absolute CD4 count.

The DP FCM methods are usually less accurate as compared with the SP FCM methods because of variation amongst hematology analyzers (43). From the multi-center trials for the inter-laboratory variation of absolute CD4 counts, the DP FCM methods were found to have > 30% coefficient of variation (CV), while the SP FCM methods showed a CV of < 17% (44).

The hematological total leukocytes count is much more accurate than the hematological total lymphocytes count (45). Recently, the traditional DP FCM methods were modified by using total leukocytes counts instead of the total lymphocytes counts from a hematology analyzer and the percentage of CD4+ T lymphocytes in total leukocytes obtained by FCM. In the FCM the total leukocytes are identified by a pan-leukocyte-reactive antibody, CD45. This new protocol is the core of the PanLeugating method (46). The accuracy has improved greatly in this way. This new approach makes a major impact globally since approximately half of laboratories still use the DP FCM methods.

It should be noted that the DP FCM methods can be updated to the SP FCM methods by adding a known number of micro-beads to each sample testing tube. The number of cells/μl is then obtained by: number of cells/μl = number of cells counted / concentration of beads / number of beads counted. The calibration beads are expensive but proved to provide good results. The current widely used formulations of calibration beads are e.g. Beckman-Coulter FlowCount micro-beads suspension, Becton Dickinson (BD) TruCount tubes and BD FACSCount dedicated reagents. A study found that inter-laboratory CVs were 12.7%, 4% and 4.6% for FlowCount, TruCount and FACSCount methods, respectively (47).
Another type of the SP FCM method is the volumetric method. This method strictly defines the ratio between the original and final sample cell concentration for absolute cell counting and requires stained and lysed blood samples in a known final volume. This method seems to be the most reliable, based on the lowest inter-laboratory variations of CD4 counts observed by enumerating stabilized cell preparations. For example, the CV of the CytorionAbsolute method was found to be only 3.2% (47, 48). The instrument of this method (Ortho, Raritan, NJ) was manufactured between 1990 and 1996 as the first bench-top FCM equipped with a precise volumetric pump for sample aspiration and delivery. However, it is not marketed anymore.

The state-of-art simple SP FCM for CD4 and CD8 enumeration

Recently several simpler and less expensive SP FCM technologies dedicated for CD4 and CD8 enumeration for HIV monitoring have been developed and evaluated.

Microbead-based technologies:

The FACSCount (BD, USA) is the only available microbead-based automated SP instrument that is designed specifically for enumerating the absolute CD4+, CD8+ and CD3+ T lymphocytes counts in a no-lyse, no-wash whole blood method. This system has been approved by many international organizations as one of the gold standards, and it has been successfully used in rural settings of many developing countries (49 - 52).

Volumetric technologies:

The Partec CyFlow volumetric system for CD4 and CD8 enumeration (Partec GmbH, Germany) is a portable, ultra compact desktop SP FCM, equipped with a single 532 nm green solid-state laser for one fluorescent color. It is designed for use in resource poor settings, capable to be powered by a 12 V car battery and/or solar panels. It applies the modern concept of primary CD4 gating (53) and a no-lyse, no-wash protocol. The instrument is relatively cheap and the cost of reagents is 5 - 20 times cheaper than that for FACSCount or other conventional FCM methods. The system has been proven to have a
good correlation with the CD4 counts obtained with the conventional FCM methods (54 - 56).

The Guava personal cell analyzer (PCA) (Guava Technologies, USA) is a micro-capillary based volumetric cytometer that applies a two colors staining protocol (with the inexpensive Guava EasyCD4 and EasyCD8 reagent kit) for CD4+ T, CD8+ T and CD3+ T lymphocytes enumeration. The instrument is highly compact and portable and without need for the large volumes of sheath fluid used in the conventional FCM. A test requires only 10 µl of whole blood and 10 µl of reagent, which reduces the test cost to a much more affordable extent. This system showed good correlation with the conventional FCM methods (57).

Hematological cytometric like method

The PointCare AuRICA (Gold Resonant Immuno Cytometry Analyzer) system (PointCare Technologies, USA) is a newly developed portable FCM system combining CD4+ lymphocyte counting and hematology testing. It is so far the only completely automated and operator-independent system for CD4+ lymphocytes enumeration. It utilizes a novel non-fluorescence beads method for the identification, classification, and counting of cells and is based on light scatter. The CD4+ T lymphocytes are identified by using CD4 conjugated gold nanoparticles, which leads to scattered light distributions that are characteristic and allow distinguishing CD4+ T lymphocytes from other cell types including CD4+ monocytes (58). This new system has not been widely evaluated yet.

Non-cytometric methods

FCM instruments are expensive and the price of an FCM assay is relatively high. Although dedicated CD4 flow cytometry systems are less expensive, the costs of the instruments and the assays are yet not affordable for resource-constrained countries. Furthermore, the operation and maintenance of an FCM requires well-trained technicians and stable electricity. Alternative non-cytometric methods have been proposed for CD4 and CD8 enumeration.
Chapter 1

Microbeads manual methods

The Coulter Manual CD4 Count kit (Beckman Coulter, USA) uses cytospheres (latex beads coated with CD4 antibody) to bind the CD4+ T lymphocytes and form a cell sphere rosette. Monocytes are identified by binding to smaller spheres coated with CD14 antibodies. Some studies suggested that this method is a useful alternative to the FCM method in resource-poor setting (59 - 61).

The Dynabeads T4-T8 quantitative system (Dynal Biotech, USA) is based on immuno-magnetic isolation of target cells from whole blood. The whole blood is first depleted of monocytes using CD14 conjugated magnetic beads, and then the target cells are isolated using CD4 and CD8 conjugated magnetic beads. The isolated target cells are stained with gentian violet and trypan blue and counted manually by light microscope or by an automated cell counter. Good correlation between this method and the FCM methods was reported (52, 62). Recently this method has been validated in resource poor settings in Africa (63, 64).

The above mentioned two micro-beads separation methods are cheap, requiring only a microscope, a hemacytometer and a manual counter; however, they are labor-intensive and may have high operator-to-operator variations.

ELISA technology

The Capcellia CD4/CD8 Test method (BioRad Laboratories, France) is a one-step immuno-enzymatic assay based on the capture of T lymphocytes by CD2 antibodies immobilized on the wells of a micro-titer plate, followed by detection with CD4 or CD8 conjugated peroxidase in an ELISA format. The CD4+ and CD8+ T lymphocytes counts are obtained by conversion of the absorbance values at 450nm using standard curves (61, 65).

The Zymmune CD4/CD8 cell monitoring kit method (Intracel, USA) uses a mixture of antibody coated magnetic and fluorescent beads. The magnetic beads isolated the cells of choice and the fluorescent beads provide the signal to count the cells (66, 67).
The TRAx CD4 Test kit method (T Cell Diagnostics, USA) is a test based on solubilization of CD4 cells from whole blood by a lysis solution to release the CD4 molecules, followed by its detection in an ELISA format (52, 51, 67 - 69).

The Elisa systems can be used in 96 well formats and be easily automated for a large number of samples. However, the methods are too complicated and have a poor correlation with the FCM methods.

Microchip technology (LabNow, USA)

Recently, a miniaturized CD4 counting system was reported, which is a microchip-based method designed for HIV monitoring in resource-poor settings. In this method, blood cells are stained with CD3 and CD4 fluorescent antibodies, captured on a membrane in a miniaturized flow cell, and then imaged by microscope optics (70). This system is expected to be marketed soon.

Table 2. A comparison of CD4 enumeration methods.

<table>
<thead>
<tr>
<th>Type of assay</th>
<th>Method</th>
<th>Manufacturer</th>
<th>Equipment cost (US$)</th>
<th>Location of use</th>
<th>Assay cost (US$)</th>
<th>Sample volume (μl)</th>
<th>Pipette steps</th>
<th>Time to result (min)</th>
<th>CD4, CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCM</td>
<td>FACSCalibur</td>
<td>BD</td>
<td>75,000 – 125,000</td>
<td>Central reference lab</td>
<td>5-80</td>
<td>100</td>
<td>&gt;3</td>
<td>30</td>
<td>both</td>
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<td></td>
<td>EPICS</td>
<td>Coulter</td>
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<tr>
<td></td>
<td>Ortho-Cytoron</td>
<td>Ortho Diagn</td>
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<tr>
<td>Dedicated FCM</td>
<td>FACSCount</td>
<td>BD</td>
<td>20,000 – 75,000</td>
<td>District/regional facility</td>
<td>2-10</td>
<td>50</td>
<td>3</td>
<td>30</td>
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<td></td>
<td>Guava PCA</td>
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<tr>
<td>Dedicated Microbeads</td>
<td>Coulter CD4</td>
<td>Coulter</td>
<td>2,000</td>
<td>District/regional facility</td>
<td>8</td>
<td>100</td>
<td>&gt;3</td>
<td>30-45</td>
<td>both</td>
</tr>
<tr>
<td></td>
<td>Dynabeads T4-T8</td>
<td>Dynal Biotech</td>
<td></td>
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<tr>
<td>ELISA</td>
<td>Cytoflex CD4/CD8</td>
<td>BioRad</td>
<td>15,000</td>
<td>District/regional facility</td>
<td>28</td>
<td>100</td>
<td>75</td>
<td>35</td>
<td>both</td>
</tr>
<tr>
<td></td>
<td>Zymmun CD4</td>
<td>Intracel</td>
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<td>TRAx CD4</td>
<td>T Cell Diagnostic</td>
<td></td>
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<tr>
<td>Microchip</td>
<td>Microchip digital imaging</td>
<td>LabNow</td>
<td>&lt; 5,000</td>
<td>Point-of-care</td>
<td>unknow n</td>
<td>&lt;10</td>
<td>None</td>
<td>10</td>
<td>both</td>
</tr>
<tr>
<td>ICM</td>
<td>StarCount</td>
<td>Twente University</td>
<td>3,000</td>
<td>Point-of-care</td>
<td>3</td>
<td>100</td>
<td>3</td>
<td>45</td>
<td>both</td>
</tr>
</tbody>
</table>
Image cytometer (StarCount, Twente University, The Netherlands)

About 5 years ago when we started this project, there was nearly no state-of-art simple SP FCM methods available. Instead, expensive conventional FCM methods and labor intensive and un-accurate microbeads manual methods were applied for CD4 enumeration. We re-investigated the concepts of improving the technology of microscopical immuno-fluorescence technologies with the aim of developing a system with advantage over the available methods. The instruments should be designed as a low-cost (as cheap as a normal microscope), compact, yet reliable, easy-to-use and robust one, suited for use in resource-poor settings. No highly qualified personnel should be needed for the preparation of samples and handling of instruments. The instruments will be computer-controlled and can operate on a 12V rechargeable battery. The output should be directly calculated in absolute CD4\(^+\) T lymphocytes count and/or CD8\(^-\) T lymphocytes count and CD4/CD8 ratio within a few min after insertion of the sample chamber into the instrument. The results of the patients must be available in about 40 - 60 min after blood draw. The throughput should be such that one well-trained operator can carry out about 50 tests with one instrument in 8 hrs.

To achieve these demands, we have developed three StarCount lines of simple single platform image cytometers (SP ICM), mainly for CD4 and/or CD8 enumeration. 1. StarCount 1.0 (SC 1.0) (one MAb, one DNA dye): for absolute leukocytes (CD45), T lymphocytes (CD3), and B lymphocytes (CD19) enumeration applying immuno-magnetic selection and nuclear staining with acridine orange (71). 2. StarCount 2.0 (SC 2.0) (two MAbs, one dye) and StarCount 2.1 (SC 2.1) (three MAbs, two dyes): for CD3\(^+\)CD4\(^+\) T lymphocytes only or for both CD3\(^-\)CD4\(^+\) T and CD3\(^+\)CD8\(^+\) T lymphocytes enumeration applying immuno-magnetic selection in combination with CD4 and CD8 immuno-fluorescent labeling (72, 73). 3. StarCount 3.0 (SC 3.0) (one MAb, two dyes): For CD4\(^+\) T lymphocytes enumeration applying CD4 immuno-magnetic selection and combining a cell activation strategy to specify CD4\(^+\) T lymphocytes. Figure 3 shows the overview of StarCount configurations, the labeling schemes and photos of instrument prototypes.
1.5 Outline of the Thesis

This thesis describes the development and the testing of our Single Platform Image Cytometer StarCount lines. In chapter 2, the immuno-chemistry methodology and instrumentation are introduced. The first system, StarCount 1.0, for absolute leukocytes (CD45), T lymphocytes (CD3), and B lymphocytes (CD19) enumeration applying immuno-magnetic selection and nuclear staining is described in chapter 3. In chapter 4, the StarCount 2.0 system, a dedicated absolute CD4$^+$ T lymphocytes enumeration system using immuno-magnetic and immuno-fluorescent staining technology is discussed. The further development of SC2.0 leads to StarCount 2.1 system for CD4 and CD8 enumeration; this new system is extensively discussed in chapter 5. In Chapter 6, the StarCount 3.0, a novel and affordable CD4 enumeration method that combines immuno-magnetic selection using only one CD4 antibody and applies a cell activation strategy is described. Chapter 7 discusses the results of a big clinical trial in Siriraj hospital in Bangkok, Thailand. The StarCount 2.1 system was compared with the gold standard FACSCount method and the dual platform FCM method. In chapter 8, we present a novel technology of building up a CD3$^+$ T cell immobilization platform for CD4 and CD8 enumeration. Finally in chapter 9, the main conclusions of the thesis are summarized and future perspectives are given.
Chapter 1

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Introduction


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62. Carella AV, Moss MW, Provost V, Quinn TC. A manual bead assay for the determination of absolute CD4+ and CD8+ lymphocyte counts in human


2.1 Introduction

In this thesis we present the results of a project that aims at the development and testing of a method for counting well-defined populations of white blood cells in resource-poor countries. The goal is to develop a prototype Image Cytometer (ICM) and the dedicated immunochemical method for the selection and recognition of the target cells.

The results we present are the output of a project that has been executed over a number of years. During these years we built several different instruments and tested a number of immunochemical procedures. Tests were performed using blood samples of healthy donors, mostly students at University of Twente, blood samples of non-HIV infected patients and blood samples of HIV infected patients from the MST hospital in Enschede. At the end of the project a test was done on a large number of HIV infected patients in the Siriraj hospital in Bangkok.

We continuously employed new principles and new components in instrument and immunochemistry. To determine whether the introduced changes improved the counting of the target cells, mainly CD4+ T lymphocytes, we compared to FCM, first on blood samples of the healthy volunteers and next, if the results were positive, on blood samples of HIV negative patients, and then on blood samples of HIV+ patients. Because of the continuous introduction of new elements many different instrument versions existed over the years. Rather than presenting all elements separately, we present in different chapters the results obtained with the instruments given in Figure 3 of chapter 1. In each chapter details of the specific instrument and method are given. In this chapter we will give a

more detailed description of the general immunochemical method and one of the instruments used, the SC2.0. This is the first prototype that reliably gave good CD4 counts on patients.

In the different cell enumeration systems three steps can be distinguished:

1. *Incubation with reagents:* In this step the whole blood, drawn from the patient, is immuno-magnetically and fluorescently labeled and diluted.

2. *Magnetic separation of target cells:* The labeled cell sample is introduced in a chamber and placed in a MagNest® (Immunicon Inc., USA). The magnetic field gradient forces immuno-magnetically labeled cells to move to the upper-surface of the chamber.

3. *Fluorescent imaging and counting:* The fluorescently labeled cells collected at the upper-surface of the chamber are imaged on a CCD. The image is then analyzed by an automatic algorithm, with or without interference of an operator.

Figure 1 shows a schematic of the sample preparation and measurement procedure. The whole procedure was designed to be simple and easy, even for less-trained personnel. It uses a no-lyse, no-wash procedure and requires minimal pipetting steps. The amount of reagents consumed is kept small to keep the test cost low.

*FIG. 1.* Schematic illustration of sample preparation and measurement procedure.
2.2 Immunochemical Methods

Incubation

For convenience and the simplicity of devices needed for sample preparation, the incubation steps are performed at room temperature (RT). The incubation times for immuno-magnetic and immuno-fluorescent labeling are kept at 15 minutes, in agreement with regular protocols for immuno-labeling of leukocytes in whole blood. This time period is the same as that used for immuno-fluorescent labeling in FCM.

Fluorescent labeling

In different SC systems we used different fluorescent labels. In the SC 1.0, the nucleus staining dye acridine orange (AO) is applied. In the SC 2.0, immuno-fluorescent label, either CD3PE or CD4PE, is applied. In the SC 2.1, a combination of CD4PE and CD8PerCP is used. In the SC 3.0, a combination of the DNA dye Hoechst33342 and Rhodamine123 (detector of reactive oxygen species (ROS) produced in an oxidative burst process showing activated monocytes and activated granulocytes) are applied.

In the SC 1.0, AO is used for cell staining. AO is a nucleic acid selective fluorescent dye. It is cationic, cell-permeable, and interacts with DNA and RNA by intercalation or electrostatic attractions. When bound to DNA, the maximum excitation is at 502 nm and the emission peak is at 525 nm (green), very similar to the spectra of fluorescein. When associated with RNA, the excitation maximum shifts to 460 nm (blue) and the emission peak shifts to 650 nm (red). AO is cheap and its spectra allow an easy excitation by LEDs and high quality separation of the emitted fluorescence by optical filters.

The bright green fluorescence of AO is applied for cell detection in the SC 1.0. The amount of acridine orange was tested by adding respectively 40 nmol, 4 nmol and 0.8 nmol AO to 100 µl of whole blood. At all concentrations tested, AO stains cells almost instantly and gives very bright green fluorescence. For the SC 1.0 we chose the amount of 0.8 nM (10 µl of 0.08 mM AO), since it was found that the more AO was used, the higher the background of the image became. Also AO tends to accumulate at the lines of free magnetic particles that form at the surface under the influence of the magnetic gradient. A
high intensity of the background and/or of the magnetic particle lines makes the
distinction of cells by the image analysis algorithm more difficult.

In the method we therefore add 10 μl of 0.08 mM AO to the 100 μl whole blood sample
immediately after the 15 min immuno-magnetic labeling and dilute with system buffer
(Immunicon Inc., USA).

Sample chamber

The analysis chambers selected are standard disposable Immunicon chambers. We
tested chambers with heights of 1.5, 2.5 and 4.0 mm, which fit directly in the MagNest®
(see below). The lower the height of the chamber, the faster the magnetically labeled cells
reach the surface. This leads to a shorter magnetic separation time. However, another
factor is also relevant with respect to the choice of the height of the chamber. The lower
the height of chamber is, the lower the amount of cells that are collected at the surface is.
This inevitably leads to poorer sampling statistics. Therefore, the height should not be too
small. In the end chambers with dimensions of 30 mm (L) × 2.7 mm (W) × 4 mm (H)
were chosen for the final design. Each chamber contains about 324 μl.

Dilution factor

The dilution factor of the blood sample is another important parameter in the
experiment design. On one hand, the higher the dilution factor, the less volume of whole
blood and reagents is needed, and the lower the cost is. On the other hand, the higher the
dilution factor, the smaller the imaged volume of whole blood is, and the poorer the
sampling statistics are. Since the chamber needs to be filled with at least ~ 324 μl of
sample, a sample preparation with a final volume of ~ 400 μl is necessary.

The optics of the SC 1.0 images 1.85 mm² (1.53 mm × 1.21 mm) from the
upper-surface of the chamber on the CCD of the smart camera. Up till about 3600 cells in
the CCD image (1300×1030 pixels) the algorithm functions correctly without delivering
too much loss due to overlapping cells. Taking into account Poisson statistics, a dilution
factor of 4.1 using 100 μl of whole blood was chosen. In this way, the imaged volume
corresponds to about 1.8 μl of whole blood. This results in a Poisson variation of 10.5% in
the concentration of cells present in whole blood at a concentration of 50/µl. This dilution factor allows accurate counting of cell populations at concentrations of 2000/µl and lower in whole blood, without losing too many cells due to cell-cell overlap. (Note that in the following development of SC lines, the dilution factor changes from 4.1 times to 4 times.)

**Immuno-magnetic particles**

We tested several different commercial immuno-magnetic particles products for use in the SC: MACS CD4 MicroBeads (Miltenyi Biotec, Germany), Dynabeads M-450 CD4 (Dynal Biotech, USA), CD4FF (CD4 conjugated ferrofluid, Immunicon Inc., USA), and EasySep Human CD4 selection (StemCell Technologies, Canada). In our MagNest® the cells labeled with MACS CD4 MicroBeads could not reach the upper-surface of the chamber due to the small magnetic moment of the beads. The cells labeled with Dynabeads M-450 CD4 reached the surface within 2 minutes. However, the target cells in the image could not be counted accurately due to the interference with the big Dynabeads (4.5 ± 0.2 µm). CD4FF from Immunicon appeared to have a high non-specific binding to other cells especially above [CD4FF] ~ 1.25 µg/ml. EasySep Human CD4 selection particles have the same size as CD4FF particles (~ 170 nm) and showed similar magnetic properties, and less non-specific adhesion; also the aggregation of these particles is less than that of CD4FF particles. EasySep magnetic nanoparticles were therefore a good alternative choice for application in our cell enumeration system.

Different from CD4FF, the EasySep magnetic nanoparticle is not conjugated to the antibody. The antibody is a tetrameric complex with an anti-cell receptor and an anti-dextran receptor. This anti-dextran receptor binds to dextran on the EasySep magnetic nanoparticle surface. The labeling needs two steps, first 15 min incubation of whole blood with antibody cocktail, then followed with another 10 min incubation of sample with EasySep magnetic nanoparticles.

We first selected EasySep for the separation step. However these beads did not function reproducibly, depending on the batch obtained. As later on the quality of CD4FF improved greatly and the non-specific binding was reduced, we performed the tests on the SC 2.0 and further systems using FF particles.
Chapter 2

Magnetic separation

To determine the magnetic separation time needed for each type of magnetic nanoparticles and to determine the appropriate amount of immuno-magnetic label needed for immuno-magnetically selecting specific type of cells, titration experiments were performed using the SC 1.0 instrument. During the magnetic separation, immuno-magnetically labeled target cells move to the upper surface of the chamber, while other cells (the rest of the leukocytes and red cells) sediment to the bottom by the influence of gravity. The principle of immuno-magnetic separation is illustrated in Figure 2.

**FIG. 2.** Schematic drawing of the immuno-magnetic separation inside the MagNest®.

Figure 3 (A, B) shows an example of the kinetics of the magnetic separation and titration of CD4FF and EasySepCD4 amount. The number of cells counted by SC 1.0 increases as a function of magnetic incubation time, the amount of CD4FF applied, and the volume of applied EasySep CD4 antibody cocktail. The ratio of the added volumes of CD4 cocktail and magnetic nanoparticle solution was set to be 2:1, as recommended by the supplier (1). Both CD4FF and EasySep CD4 showed a long plateau when sufficient reagents were applied. The amount of cells at the plateau shows that not all cells reach the surface. This is due to the CD4$^{+}$dim monocytes that do not have sufficient CD4 receptors to bind enough magnetic labels and therefore could not reach the surface. For CD4FF, the larger plateau value when larger amount of CD4FF was used could indicate that more CD4FF particles induced more non-specific binding. For EasySep CD4, the value of the
plateau is independent of the volume of the cocktail applied, indicating very low non-specific adhesion.

**FIG. 3.** Magnetic separation kinetics and titration of CD4FF (A) and EasySepCD4 (B). The number of cells per μl counted by SC 1.0 is plotted as a function of magnetic incubation time and the amount of CD4FF or the volume of EasySepCD4 antibody cocktail used. Error bars in each dot represent the square root of the value. This blood sample has 715 CD4⁺ T lymphocytes/μl and 412 CD4⁺ dim monocytes/μl (FCM data).

The magnetic separation time in our SC systems using FF was chosen to be 20 min. In the experiments with different versions of SC systems, the immuno-magnetic labeling and separation conditions were determined according to separate titrations. The development of the immuno-chemical methods for each different StarCount system will be described in the appropriate chapters.

Table 1 gives an overview of the experimental settings used in different systems.
### Table 1. Experimental parameters for different StarCount Image Cytometers

<table>
<thead>
<tr>
<th>SC1.0</th>
<th>Magnetic label</th>
<th>Fluorescent label</th>
<th>Dilution factor</th>
<th>Reagents incubation time (min)</th>
<th>Magnetic separation time (min)</th>
<th>Image volume (μl WB)</th>
<th>Poisson variation at 50 cells/μl (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EasySep CD45, CD3, CD19</td>
<td>Acridine Orange</td>
<td>4.1</td>
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<td>20</td>
<td>1.8</td>
<td>10.5</td>
<td></td>
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<td>EasySepCD3or EasySepCD4; CD3FF or CD4FF</td>
<td>CD4PE or CD3PE</td>
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<td>20</td>
<td>0.80 × 3</td>
<td>9.1</td>
</tr>
<tr>
<td>SC2.1</td>
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<td>CD4PE &amp;CD8PerCP</td>
<td>4</td>
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<td>20</td>
<td>1.16 × 3</td>
<td>7.6</td>
</tr>
<tr>
<td>SC3.0</td>
<td>CD4FF</td>
<td>Hoechst &amp; Rhodamine</td>
<td>4</td>
<td>15</td>
<td>20</td>
<td>1.10 × 3</td>
<td>7.8</td>
</tr>
</tbody>
</table>

### 2.3 StarCount Instrumentation

#### Requirements

The design criteria resulting from the applications envisaged, are that the instrument has to be cheap, so affordable components should be used, yet it must have a high sensitivity, good selectivity and a reliable performance. In addition, the instrument should be easy-to-use even for less-trained personnel. It should be portable, have low power consumption and should be able to operate stand-alone on a rechargeable battery. It should also have a good stability and require little maintenance.

#### Functional design

The functional design is similar for all of our SC instruments and is illustrated in Figure 4. Where appropriate we use the SC 2.0 as an example.
The instrument is an automatic ICM that takes a fluorescent image of immuno-fluorescently labeled cells (CD4$^+$ T lymphocytes in case of the SC 2.0) that have been magnetically collected at the upper-surface of a sample chamber inserted between the poles of a magnet. Light Emitting Diodes (LEDs) are used as excitation light sources. Excitation filters suppress light emitted by the LEDs in the spectral region that overlaps with the fluorescence emitted by the cells. Custom optics collimates the LED output on the analysis area of the sample chamber. A standard microscope objective collects the fluorescence emitted by cells onto a CCD camera. The objective is mounted on a stage (not shown) to be adjusted for focusing. An emission filter selects the fluorescent signal. The acquired image is transferred to a single board computer (SBC) that is operated by a touch-screen monitor. The image is analyzed using a dedicated image analysis algorithm to determine the number of CD4$^+$ T lymphocytes per µL of whole blood. The CCD camera, LED units, the SBC and the touch-screen are connected to a 12 V rechargeable battery via a custom-built electrical circuit. All components, except the battery are enclosed in a mechanical frame (not shown), while the battery is in a separate frame.
Factors determining the sensitivity

A reliable counting requires images with a high signal-to-noise ratio (SNR). A high fluorescence signal of the (fluorescently labeled) cells can be obtained by using a high illumination power (high-power LEDs and/or more LED units), a long illumination time and high collection efficiency. The LED output should be efficiently collimated by the optics on the measuring area of the sample chamber at a high intensity and with a homogeneous distribution.

An important aspect is to keep the background as low as possible. The background is mainly caused by residual unbound fluorophores in the sample, auto-fluorescence from the cells, reflected- and stray LED light that passes through the filter, and room light. Use of proper excitation and emission filters will suppress part of these factors, but can not reduce the background induced by the free fluorophores. Another source of noise is the CCD camera that generates dark current noise, readout noise and thermal noise. Noise may also be caused by the variation in sensitivity of separate pixels of the CCD chip.

Illumination unit

A schematic drawing of an assembled LED illumination module is shown in Figure 5. Each Luxeon V Star LED (5 W, 470 nm, LXHL-LE5C, Lumileds, USA) unit requires a typical operating voltage of 6.84 V @ 700 mA and outputs a luminous flux of 48 lumen (lm). A fiber coupling lens with a focal distance of 16 mm (Roithner Lasertechniek, Austria) collimates the output of each LED onto the surface of the sample chamber. The LED and lens are assembled in a lens holder (Fraen Corporation, USA). A 475AF50 excitation filter (Omega Optical, USA) is positioned in front of the lens to suppress the LED output overlapping with the fluorescence emitted by labeled cells. All these optical components are aligned and packed together by a 25 mm shrink tubing (Farnell, UK). The three legs of each module are heat staked into a metallic plate that serves also as a heat sink. In this way an optical assembly with high mechanical stability is obtained.

Four LED modules are mounted symmetrically on top of the magnetic yoke, with the optical axis at a 45 degrees angle with respect to the plane of the stage. The 45 degrees was optimal to obtain a maximum light intensity and a relatively good uniformity on the
surface of the sample chamber. The distance between each LED module and the chamber surface can be adjusted. The optimum distance is ~20 mm. The diameter of the LED beam at the top of the chamber is ~8 mm. The overall light intensity on the measuring area is 3.84 lm/mm$^2$ (four LED units), with a CV of <10%, as determined by WinCamD beam diagnostics (Gentee-EO Inc., Canada).

**FIG. 5.** A schematic drawing of the LED module.

**Imaging optics**

A 10× microscope objective (NA 0.2) (Lomo Optics, USA) is mounted on a stage that enables adjustment of the focus that can be locked. This objective is applied to obtain a magnification of 6.8 times. The PE fluorescence, emitted by labeled cells, is filtered by a 595AF60 emission filter (Omega Optical, USA). The CCD covers a field of view (FOV) of 1.09 mm × 0.73 mm of the upper-surface inside the chamber, which corresponds to 0.80 μl of whole blood (FOV is multiplied by the height of the chamber and then divided by the dilution factor).

Figure 6 illustrates that the spectrum of the LED matches well with the absorption spectrum of PE. A higher yield could be achieved by a LED with an output wavelength of 505 nm or 530 nm, but that is not suitable in this optical configuration. It would result in a higher background in the image due to a substantial interference of the LED light with the emission of PE caused by the broad emission spectrum of the LED (The spectral width at half maximum intensity is 30 nm).
FIG. 6. Emission spectrum of the 470 LED (dark blue), absorption (light blue) and emission (pink) spectra of PE, and transmission spectra of the 475AF50 excitation filter (green) and the 595AF60 emission filter (red).

On the CCD, each fluorescently labeled CD4+ T lymphocyte is shown as a spot of approximately 25 pixels. The number of pixels used to image one cell is an important parameter. On one hand, the accuracy is increased with more pixels per cell, since the resolution increases and it leads to an easier separation of two neighboring cells in an image. However, this induces a decrease in the SNR when the light intensity per pixel is compared with the noise in surrounding pixels. On the other hand, the larger the number of pixels used per cell, the fewer cells can be imaged on the CCD surface, which will lead to a poor sampling statistics. 25 pixels per cell is a reasonable compromise.

CCD camera
The CCD camera (ST-402ME; SBIG, USA) was designed for astronomical purposes where the faint details of dim astronomical objects are imaged. Similarly, we apply it to image the weak immuno-fluorescence of labeled cells. This camera offers a high performance at relatively low costs. The imaging CCD chip (Kodak KAF-0402ME imaging CCD; Kodak, USA) has 765 × 510 pixels (9 μm × 9 μm per pixel), and combines
Experimental Methods and Instrumentations

A large well capacity (~100,000 e−) with a low dark current (1 e−/pixel/sec at 0 °C) and a low readout noise (17 e− RMS (the square root (R) of the arithmetic mean (M) of the square (S) of density variations)). The camera has a 16 bits A/D converter. Due to the implementation of a micro-lens array over the pixels, it has high quantum efficiency (~75% at the peak of PE emission). Dark image subtraction can be performed for images acquired with exposure times longer than 1 sec to remove the noise caused by CCD. The camera operates on a 12 V battery or on any other unregulated 12 V DC source.

SBC and touch-screen

The cell images on the CCD camera are uploaded to a Single Board Computer (SBC). The SBC is used for images collection, analysis and overall instrument control. It is a Micro PC with a VIA Eden™ ESP6000 667 MHz processor (EES-3610, Evalue Technology, Taiwan), a 256 MB SDRAM SODIMM memory (SimpleTech Inc., USA) and a 2.5” hard disk (Toshiba, Japan). The Micro PC is a low-power fan-less embedded system (operating with 5 V DC @ 5 A) with dimensions of 220 mm × 65 mm × 147 mm and a weight of 1.7 kg. It is inexpensive compared to a normal computer or a laptop. Furthermore, its size, weight and power consumption allow development of a stand-alone platform.

An 8.4” LCD-TFT touch-screen monitor (B084SN03 V2, AU Optronics, Taiwan) with a touch-screen controller (JTHC084DRA1, AU Optronics, Taiwan) are connected to the SBC. The touch-screen is powered by a 12 V voltage.

Power supply and housing

The total power consumption of this ICM is 55 W, from which about half is consumed by SBC and touch-screen. To minimize power consumption, the LEDs are controlled via a relay card (USBREL8LC, Quancom, Germany) and operate only during the image acquisition (10 seconds per image). A home built power unit contains one 12 V rechargeable battery with a capacity of 7 Ah (NP 7-12, Yuasa, Japan), that is charged by a 12 V/1 A battery charger (LM12010-3T, MEC Ltd, Hong Kong). The ICM needs 5 V for the SBC, 6.8 V for the LED units and 12 V for the CCD camera and LCD-TFT.
touch-screen. Two voltage regulators (LT1083, Linear Technology, USA) output 5 V and 6.8 V. The power supply is connected via a five pole connector to the ICM.

With a fully charged battery, the current prototype runs for about 2.5 hrs. In this time, ~25 samples for CD4 enumeration can be analyzed. In case of power loss, an external car battery can be connected to the power unit. A 30 Ah battery can last for about 10 hrs operation.

A home-built preliminary aluminum housing (25 cm × 25 cm × 20 cm) accommodates all components, except for the power unit. The optical parts, including CCD and magnetic yoke, are positioned at the front part of the instrument. The SBC is mounted on the rear vertical plate that serves as heat sink. The power unit, built in a separate housing, is mounted on the outside of the rear plate. The touch-screen is positioned on top of the cytometer under an angle of 45 degrees. Total weight of the ICM is 15 kg, and 5 kg from it is the weight of the power unit.

**Image analysis**

To obtain absolute CD4^+ T lymphocytes counts, fluorescent images were analyzed using a dedicated algorithm written in ImageJ, a public-domain Java image processing software package (NIH, Maryland, USA) (2). The algorithm consists of the following steps: 1. **Background subtraction**: To correct non-uniform illumination, variation in the sensitivity of the CCD pixels, etc. 2. **Thresholding**: To transform the grey-scale image to a binary image. All the objects (cells) that have higher intensity than a pre-set threshold are assigned as 1, and the rest of the objects that have lower intensity (background) are assigned as 0. 3. **Watershed**: To separate the overlapping cells to mark the boundaries of each single object (cell) by determining the center of a cell (peak intensity) followed by dilating until the edge of a cell or the edge of an adjacent cell is reached. 4. **Counting**: To enumerate all objects (cells) assigned as 1. 5. **Separation**: An additional step that first estimates the average area of a cell in the image and then divides the total area of overlapping cells by this average cell area to obtain the cell count. The **Watershed** and the **Separation** steps are necessary for images with a high cell count to allow a better separation of the overlapping cells.
All steps of the algorithm are implemented in a macro that enables an automatic image analysis. Analysis of one image takes less than 10 seconds in the SBC.

**FIG. 7.** Typical PE fluorescence image of CD4⁺ T lymphocytes obtained with the SC 2.0 instrument prototype (A); this image after applying a background subtraction (B) and thresholding (C); a merge of the original image with red circles that indicate cells that are selected by the image analysis algorithm (watersheding) (D). (E, F, G and H): Enlarged images from the selected area in A, B, C and D, respectively.
Figure 7 illustrates the image analysis process. A typical image of CD4+ T lymphocytes labeled with CD4PE is shown. Results of background subtraction, thresholding and watershedding are also presented. In this case apparently all cells have been selected by the image analysis algorithm.

Software shell

To perform measurements in an easy and reliable way, a software shell (LabView, National Instruments, USA) that integrates all hardware and software into one graphical user interface (GUI), has been developed. The measurement cycle is started by pressing a "START" button. Then a pop-up screen asks for the patient ID and the sample number. Next, the type of measurement has to be chosen between the manual CD4 measurement and the custom CD4 measurement. The exposure time needed for acquiring an image can also be set. Next, an image is taken, and subtracted from a dark image, and shown on the front panel of GUI. Next, the image is analyzed and a text report containing the patient information, the CD4 count and the cell image with selected circles is shown. The whole procedure from image acquisition to the result of the cell count takes less than 1 minute. If the custom CD4 mode encounters difficulties for some blood sample, the manual mode enables the user to adjust all different parameters separately to analyze once again.

It should be noted that the measurements presented in this thesis were not performed using this shell as it was too late available. The images were captured and saved by the camera related CCDOP software and later on analyzed, using the image algorithm macro with a “manual thresholding” setting, on a separate computer.

Overview of instrument settings

The instrument settings are slightly different for different StarCount systems. Table 2 gives an overview of the instrument settings for different StarCount systems. The details will be described in the following chapters.
Table 2. Overview of the instrument settings for different StarCount systems. (RCB: rechargeable battery)

<table>
<thead>
<tr>
<th>SC</th>
<th>LED</th>
<th>Excitation filter</th>
<th>Emission filter</th>
<th>Objective</th>
<th>Camera</th>
<th>SHCC&amp;Touch-screen</th>
<th>Illumination time (sec)</th>
<th>Measuring time (min)</th>
<th>Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>2x5mW (470nm)</td>
<td>no</td>
<td>515LP</td>
<td>10x (NA0.2)</td>
<td>Smart CCD w/o</td>
<td>2</td>
<td>&lt; 1</td>
<td>12V RCB</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>4x5mW (470nm)</td>
<td>4x475AF50</td>
<td>575AF60</td>
<td>10x (NA0.2)</td>
<td>ST-402 ME</td>
<td>With</td>
<td>10 × 3</td>
<td>1 × 3</td>
<td>125-240 V or 12V RCB</td>
</tr>
<tr>
<td>2.1</td>
<td>2x3W (490nm)/4x3W</td>
<td>2x4x550SP</td>
<td>595AF60&amp;695AF55</td>
<td>10x (NA0.2)</td>
<td>ST-402 ME</td>
<td>With</td>
<td>(2x10) × 3</td>
<td>2 × 3</td>
<td>125-240 V or 12V RCB</td>
</tr>
<tr>
<td>3.0</td>
<td>2x6mW (375nm)/2x3W (493nm)</td>
<td>2x470AF50</td>
<td>420LP&amp;535AF45</td>
<td>10x (NA0.2)</td>
<td>ST-402 ME</td>
<td>Laptop</td>
<td>(10 &amp; 1) × 3</td>
<td>2 × 3</td>
<td>125-240 V or 12V RCB</td>
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REFERENCES

An Immuno-magnetic Single Platform Image Cytometer for Cell Enumeration Based on Antibody Specificity

ABSTRACT
Background: Simplification of cell enumeration technologies is required especially for resource-poor countries where reliable and affordable enumeration systems are greatly needed.

Methods: In this paper, an immuno-magnetic single platform image cytometer (SP ICM) for cell enumeration based on antibody-specificity was reported. A chamber/magnet assembly was designed such that the immuno-magnetically labeled, acridine orange stained cells in a blood sample moved to the surface of the chamber, where a fluorescent image was captured and analyzed for cell enumeration. The system was evaluated by applying one kind of antibody to count leukocytes and one kind for each leukocyte subpopulations: CD45 for leukocytes, CD3 for T lymphocytes, and CD19 for B lymphocytes. Excellent precision and linearity were achieved. Moreover, these cell counts, each from blood specimens of 42 to 52 randomly selected patients, were compared with those obtained by single- (TruCount) and dual-platform (DP) flow cytometry (FCM) technologies.

Results: The cell counts obtained by our system were in between those obtained from the TruCount and the DP FCM methods; and good correlations were achieved (R ≥ 0.95). For CD4+ counts, as we expected, the cell count by our system was significantly higher than CD4+ T lymphocytes counts obtained by SP and DP FCM methods. Immuno-phenotyping of the CD4+ immuno-magnetically selected cells illustrated that,
besides CD4⁺ T lymphocytes, a proportion of the CD4⁺ dim monocytes were also selected.

**Conclusion:** Our system is a simple immuno-magnetic single platform image cytometer, which can potentially be used for CD3⁺CD4⁺ T lymphocytes enumeration in resource-poor countries in case additional CD3 immuno-fluorescent label is applied.

**INTRODUCTION**

Absolute enumeration of cells in clinical samples becomes more and more important. Examples include enumeration of leukocyte and its subpopulations in blood of patients, of residual leukocytes in leukocyte-depleted blood transfusion products (1), and of circulating tumor cells in peripheral blood of cancer patients (2, 3).

Absolute cell enumeration is usually accomplished by single platform (SP) and dual platform (DP) flow cytometry methods (FCM). SP FCM methods use calibration beads (4) or employ a volumetric method (5). Dual platform (DP) FCM technologies calculate the absolute number of cells of one or more subpopulations by multiplying the absolute cell count obtained by an automatic hematology analyzer with the percentage of each specific cell subpopulation obtained by FCM (6). The DP FCM methods are usually less accurate as compared with the SP FCM methods because of variation amongst hematology analyzers (7). In general both SP and DP methods are expensive in equipment, maintenance and technician training (8). Consequently, it is of significance to develop simpler SP cell enumeration technologies.

We developed an immuno-magnetic method using a single platform image cytometer (SP ICM) to enumerate leukocyte and its subpopulations that can uniquely be characterized by one specific type of antibody. This method is realized by using the so-called ‘cellular astronomy’ technology (9), e.g. Light Emitting Diodes (LEDs) for illumination, and a smart camera for imaging and analyzing of the obtained images. In our method the target cells are immuno-magnetically labeled and fluorescently stained with acridine orange (AO). The labeled cells from a known volume of sample are then driven by a magnetic force to a surface of an analysis chamber, where these cells are illuminated using LEDs (10). A fluorescent image of the target cells at the surface is
captured by a smart CCD camera with software that counts the cells and calculates the number of cells per μl of whole blood. The method yields absolute cell counts. The system is easy-to-handle and battery-operated.

In this paper, we demonstrate the performance of our cell enumeration system in counting CD45\(^+\) leukocytes, CD3\(^+\) T lymphocytes, and CD19\(^+\) B lymphocytes in human whole blood. The immuno-magnetic selections of these cells are governed by their antibody specificity. By comparing the cell counts obtained from our SP ICM method to those from SP and DP FCM methods, the accuracy of our system is assessed. In case the immuno-magnetic label used is not specific for only one type of cell subpopulation, as for CD4\(^+\) cells, both CD4\(^+\) T lymphocytes and CD4\(^+\)dim monocytes (11) are selected. Additional immuno-labeling should be applied to accurately enumerate CD4\(^+\) T lymphocytes for HIV staging. The results are described in chapter 4 of this thesis.

MATERIALS AND METHODS

Image Cytometer Instrumentation

The instrument is in principle a simple single platform image cytometer based on an automated fluorescence microscope (Figure 1). The optical components consist of two 5 mW LEDs (Marl 110106, Nichia, Japan), a 10× objective (LOMO Optics, Germantown, MD, USA) and an emission filter (RG 540, Schott, Germany). Images are captured and processed with a smart CCD camera (1300 × 1030 pixels, VC67, Vision Components, Germany). Upon insertion of an analysis chamber into the instrument, a micro-switch starts the measuring cycle. The camera software then controls all further steps and the captured images are analyzed using a home-made image analysis program. The number of cells per μl of whole blood is displayed on a liquid crystal display (LCD). The system operates on a 12 V rechargeable battery, which can last for about 400 tests. The overall dimensions of the cell enumeration system are 17 cm × 17 cm × 19 cm (Figure 1A).

Cell Enumerations of Single Platform Image Cytometer (SP ICM)

Blood collection
Blood specimens were collected in sterile K$_3$EDTA vacutainer blood collection tubes (BD Biosciences, USA). Blood specimens from randomly selected HIV negative patients were supplied by the local hospital (Medisch Spectrum Twente, Enschede, The Netherlands) and processed within 6 hours after draw.

**Labeling of target cells**

To select leukocytes and the leukocyte subpopulations, e.g. T lymphocytes, B lymphocytes, and CD4$^+$ cells from whole blood, we labeled the target cells immuno-magnetically using the following kits (EasySep, StemCell Technologies, Canada): Human CD45 (clone: MEM28, isotype: mouse IgG1) depletion, CD3 (clone: UCHT-1, isotype: mouse IgG1) positive selection, CD19 (clone: AE-1, isotype: mouse IgG1) positive selection, and CD4 (clone: QS4120, isotype: mouse IgG1) positive selection. Each kit comprises a specific antibody cocktail and a dispersion of EasySep magnetic nanoparticles, and was used according to the manufacturer’s specifications. Acridine orange (AO; Molecular Probes, USA) was used to stain nucleated cells.

By titration, the optimal volumes of antibody cocktail and magnetic nanoparticle dispersion, concentration of acridine orange, incubation time of reagents, and magnetic separation time were determined. The optimal amounts of antibody cocktail and nanoparticle dispersions for our experiments were found to be: 5 µl of CD45 cocktail and 10 µl of magnetic nanoparticle dispersion, 10 µl of CD3 cocktail and 5 µl of magnetic nanoparticles dispersion, 12 µl of CD19 cocktail and 6 µl of magnetic nanoparticles dispersion, and 15 µl of CD4 antibody cocktail and 7.5 µl of nanoparticles, respectively.

**Cell enumeration procedure**

To 100 µl of whole blood, the appropriate volume of antibody cocktail was added and mixed. (For leukocyte enumeration, 100 µl of 20-time diluted blood sample was used). After 15 min incubation, the corresponding volume of magnetic nanoparticle dispersion was added and mixed. After another 10 min of incubation, Acridine orange (10 µl of 0.08 mM) was added and the sample was diluted with system buffer (Immunicon Inc., USA) to a final volume of 410 µl. Approximately 340 µl of the diluted sample was then transferred...
into an analysis chamber. The chamber was plugged and placed in a magnet assembly (MagNest®, Immunicon Inc., USA) as illustrated in Figure 1B. After 20 min magnetic separation, the analysis chamber was transferred into the MagNest® inside the SP ICM instrument. Fluorescent images of the analysis surface were captured and analyzed. The measurement and image analysis took about 40 s.

**Linearity**

To evaluate the linearity of our system within the low cell count range (0 ~ 400/µl), a series of diluted blood samples containing graded, known number of CD3 T lymphocytes were prepared according to the following protocol:

From a whole blood specimen of a healthy donor, a CD3 enumeration was performed by our system. Then the original whole blood specimen was diluted with system buffer in different ratios, resulting in a series of 6 specimens with known numbers of CD3 T lymphocytes (12, 25, 50, 100, 200 and 400/µl). Each diluted blood sample was performed CD3 enumeration for 5 replicates. The linearity was evaluated.

**Single Platform and Dual Platform Flow Cytometry Methods**

Both SP and DP FCM methods were used to obtain absolute cell counts as controls to evaluate the accuracy of our SP ICM method and the interchange-ability between our method and FCM methods.

For SP FCM method, the TruCount™ method was used. The blood specimens were processed using a lyse-no-wash method according to the manufacturer’s recommendation (BD Biosciences, USA), and analyzed by FACSCalibur using CELLQUEST™ software (BD Biosciences, USA) (4).

For DP FCM method, the relative proportions of specific cell subpopulations were obtained by FACSCalibur using CELLQUEST™ software. The absolute number of total leukocytes was determined by a Sysmex SE-9500 hematology analyzer (Sysmex Corporation, Japan).

For both FCM methods, the reagents used were Fluorescein isothiocyanate (FITC)-conjugated CD3 (clone SK7; isotype, mouse IgG1-κ), phycoerythrin
(PE)-conjugated CD4 (clone SK3; isotype, mouse IgG1-κ), Peridinin chlorophyll protein (PerCP)-conjugated CD45 (clone 2D1; isotype, mouse IgG1-κ), and FITC-conjugated CD3 (clone SK7; isotype, mouse IgG1-κ), PE-conjugated CD19 (clone SJ25C1; isotype, mouse IgG1-κ), PerCP-conjugated CD45 (clone 2D1; isotype, mouse IgG1-κ) (TriTEST, BD Bioscience, USA) and FACS Lysing Solution (BD Bioscience, USA).

Immuno-phenotyping of CD4⁺ Immuno-magnetically Selected Cells

To determine the blood cell populations represented in immuno-magnetically selected CD4⁺ cells, we incubated the blood samples with CD4 immuno-magnetic particles, and immuno-fluorescent labels CD3 (clone UCHT-1; isotype, mouse IgG1-κ)-allophycocyanin (APC), CD14 (clone MΦ90/P9; isotype, mouse IgG2b-κ)-PE, and CD66b (clone G10F5; isotype, mouse IgM-κ)-FITC (BD Biosciences). Acridine orange was not added in this case. After sample incubation, the sample chamber/MagNest® assembly was placed under a fluorescent microscope (Nikon ECLIPSE E400, Japan) equipped with a 40× objective (NA 0.6). Fluorescent images were taken from 10 randomly selected areas of the chamber surface using appropriate filters for FITC, PE and APC. Blood specimens from 3 healthy donors were used.

Statistical Analyses

To assess the test precision of SP ICM method, we enumerated for 27 patients five replicates of their fresh blood specimens. For each patient the coefficient of variation (CV = standard deviation/mean × 100%) of the five cell counts was calculated and the results were compared with the Poisson variation (see below).

To assess the accuracy of our method, we compared the SP ICM results with those of SP and DP FCM methods. Based on the cell counts obtained from different methods, linear regression lines were drawn and correlation coefficients (R) were calculated. Bland-Altman plots (12 - 15) were analyzed to determine the interchange-ability between the methods. In the Bland-Altman plots, the average of the cell counts obtained by the two methods is plotted on the x-axis, and the difference between the results by the two methods divided by the average, expressed as a percentage, is plotted on the y-axis. The
RESULTS

Design of Image Cytometer

Figure 1 shows the prototype of the image cytometer we built (Figure 1A), and a scheme with the separate components of the image cytometer.

For magnetic separation, the incubated blood sample is transferred into an analysis chamber with inner dimensions of 30 mm (L) × 2.7 mm (W) × 4 mm (H). The chamber (outer) top surface is placed 2 mm below two magnetic poles of the MagNest®. These poles, made of Neodymium Iron Boron, have an internal magnetization of 13.7 kGauss. The distance between the two poles is 3 mm and the top angle of each pole piece is 20° (Figure 1B). The force inside the chamber, generated by the magnetic gradient, points to the positive Z-direction of the chamber (also the axis of the objective) and has no components in the X- or Y-axis. As a result, all immuno-magnetically labeled cells move along the positive Z-direction to the upper-surface of the chamber (Figure 1B). Cells that have not been immuno-magnetically labeled sedimentate to the bottom of the chamber due to gravity. The chamber/MagNest® assembly is at 9° with respect to the horizontal plane; this is to move air bubbles out of the field of view. To eliminate the need of re-focusing at each time when a new analysis chamber is placed into the magnet, spring-loaded ball bearings are applied, which push the upper-surface of the chamber to a fixed position ensuring the sharp image of the cells.

The layout of the optical design is shown in Figure 1C. Two LEDs are placed symmetrically above the chamber, at an angle of 45° to the upper-surface of the analysis chamber to illuminate the region of interest. The maximum emission wavelength of the LEDs is 470 nm; this matches the absorption spectrum of acridine orange. The fluorescence emitted by the acridine orange-stained nucleated cells is collected by a 10× objective (NA 0.2), which is perpendicular to the analysis surface; then the fluorescence is filtered through a 515 nm long-pass filter. The image is focused on the CCD surface of
a smart camera. The camera is programmable in C++ and can be used to analyze the images. The spectra of the LED, acridine orange, and the long-pass filter are shown in Figure 1D.

**FIG. 1.** A: Prototype of the cell enumeration system. a, MagNest®; b, objective; c, CCD camera; d, LCD. B: Schematic representation of the trajectory of magnetically labeled
objects. The dashed lines inside the chamber illustrate the trajectories of the cells that move up due to the magnetic force, starting from different positions. C: Schematic representation of the optical design. D: Spectra of the LED (emission), acridine orange (AO) (excitation and emission) and the 515nm long-pass filter. E: Recorded image of cells stained with acridine orange. F: Processed image from E, red dots represent the events that have been counted as cells. G, H: Enlarged images from the selected area as shown in E and F, respectively (color figures on Page 212).

To start a cell counting cycle the analysis chamber is inserted into the instrument. A micro-switch triggers the measurement cycle. The LEDs illuminate the sample for 2 seconds and a fluorescence image is captured. Then the image is processed by the image analysis algorithm. The total time required to capture and to analyze an image, and to display the cell count per $\mu$l of whole blood on the LCD is approximately 40 seconds.

Typical images before and after analysis are shown in Figure 1E and Figure 1F, respectively. Figure 1G and 1H are the enlarged images from the selected area in Figure 1E and 1F, respectively. Each figure represents an analyzed image area of $1.85 \text{ mm}^2$ (1.53 mm × 1.21 mm). As the height of the chamber (inside distance) is 4 mm and the blood sample has been diluted 4.1-fold (or 82 fold for leukocyte enumeration), the cells shown in each image correspond to those originally present in $1.80 \mu$l of whole blood (or 20-time diluted blood sample for leukocyte enumeration).

**Evaluation of SP ICM**

**Precision**

To assess the test precision of our system, leukocytes of five replicates from each of the blood samples of 27 patients were enumerated (in total 135 measurements). From cell counts of the replicates of each blood sample, the coefficient of variation (CV = standard deviation/mean × 100%) was determined. Assuming that the distribution of the cell counts of the five replicate samples follows a Poisson distribution, the expected statistical Poisson variation of the cell count can be estimated according to the following equation:
where the cell count/μl is based on the total sampling of 1.8 μl of the 20-time diluted blood samples.

FIG. 2. CV (diamonds) and Poisson variation values (squares) of leukocyte counts from five replicates of each of the blood samples (20-time diluted) of 27 patients plotted against their mean leukocyte counts.

Figure 2 shows the Poisson variations and the CV values of each of the 27 blood samples vs. their mean leukocyte counts. The mean leukocyte counts of these blood samples (20-time diluted) ranged from 145/μl to 1712/μl; correspondingly, the Poisson variations range from 6.19% to 1.80%. The measured CV values ranged between 2.38% and 8.56% for these 27 blood samples. In most cases, the measured CV is slightly higher than the Poisson variation, indicating that the precision of our system is mostly determined by Poisson statistics, although other errors, e.g. sampling errors are present.
To evaluate the linearity of our system, a whole blood sample was performed CD3 enumeration and a series of diluted blood samples containing graded, known numbers of CD3 T lymphocytes (12, 25, 50, 100, 200 and 400/μl) were prepared. In Figure 3, the measured CD3 T lymphocyte counts were plotted as a function of the expected CD3 T lymphocyte counts of these samples. The error bar represents the standard deviation of 5 replicates of CD3 T lymphocyte counts. A linear relation (slope = 1.03; R = 0.999) was found, demonstrating the linearity of our system in the low cell count range (0 ~ 400/μl).

**FIG. 3.** Linearity of our system in low cell count range (0 ~ 400 CD3 T lymphocytes/μl). The error bar represents the standard deviation of 5 replicates of CD3 T lymphocyte counts.

**Accuracy of SP ICM in counting cells with specific antibodies**

To determine the accuracy of our method, leukocytes (blood specimens of 50 patients), T lymphocytes (blood specimens of 52 patients), and B lymphocytes (blood specimens of 42 patients) were enumerated by our method and by the SP and DP FCM methods, respectively. For enumeration of these cells by SP ICM, the immuno-magnetic selection is governed by antibody specificity, i.e., these cells are uniquely labeled using the specific...
antibodies. Table 1 summarizes the correlation coefficients (R) and the slopes of the linear regression lines, and the biases obtained from the Bland-Altman plots.

**TABLE 1. Correlations and biases among cell counts obtained by our cell enumeration system (SP ICM), TruCount, and DP data for different cell populations.**

<table>
<thead>
<tr>
<th>Cell type (antibody)</th>
<th>Leukocytes (CD45)</th>
<th>T lymphocytes (CD3)</th>
<th>B lymphocytes (CD19)</th>
<th>CD4+ T lymphocytes (CD4)</th>
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</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>50</td>
<td>52</td>
<td>42</td>
<td>45</td>
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**Linear regression**

<table>
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<th>R</th>
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<th>R</th>
<th>Slope</th>
<th>R</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICM vs. TruCount</td>
<td>0.95</td>
<td>0.89</td>
<td>0.97</td>
<td>0.86</td>
<td>0.96</td>
<td>1.01</td>
<td>0.83</td>
<td>1.42</td>
</tr>
<tr>
<td>ICM vs. DP</td>
<td>0.97</td>
<td>1.14</td>
<td>0.97</td>
<td>1.09</td>
<td>0.95</td>
<td>1.23</td>
<td>0.85</td>
<td>1.75</td>
</tr>
<tr>
<td>TruCount vs. DP</td>
<td>0.98</td>
<td>1.26</td>
<td>0.98</td>
<td>1.26</td>
<td>0.99</td>
<td>1.23</td>
<td>0.97</td>
<td>1.24</td>
</tr>
</tbody>
</table>

**Biases of Bland-Altman plot (%)**

<table>
<thead>
<tr>
<th></th>
<th>TruC-ICM</th>
<th>DP-ICM</th>
<th>TruC-DP</th>
</tr>
</thead>
<tbody>
<tr>
<td>/Average% vs. Average</td>
<td>10.1</td>
<td>10.0</td>
<td>22.0</td>
</tr>
<tr>
<td>/Average% vs. Average</td>
<td>−12.0</td>
<td>−12.9</td>
<td>22.8</td>
</tr>
<tr>
<td></td>
<td>−37.5</td>
<td>−56.4</td>
<td>20.5</td>
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</table>

First, the results obtained by our SP ICM method and those obtained by TruCount method were compared. Figures 4A-C show for the different cell populations the linear regression lines comparing the cell counts obtained by these two methods. The correlation coefficients (R) and slopes are also indicated. In parallel, Bland-Altman plots are given in Figure 5A-C. The bias between the two methods and the upper and lower limits of agreement are indicated.
Linear regressions of cell counts by our cell enumeration system (SP ICM) and TruCount for leukocytes (20 times diluted whole blood) (A), T lymphocytes (B), B lymphocytes (C), and CD4+ T lymphocytes (D). Blood specimens of 42 to 52 patients were analyzed (for details see Table 1). The error bar in each dot represents the square root of the value.

For leukocytes and T lymphocytes, our enumeration method shows a good correlation with TruCount ($R = 0.95$ and $0.97$, respectively). However, the slopes of the linear regression lines for these populations (0.89 for leukocytes, and 0.86 for T lymphocytes) show that the results obtained by our method for those populations are about 11% to 14% lower than those by the TruCount. In agreement with this observation, the Bland-Altman plots show a bias of approximately 10% (TruCount vs. SP ICM).
FIG. 5. Bland-Altman plots comparing cell counts generated by our cell enumeration system (SP ICM) and TruCount for leukocytes (20 times diluted whole blood) (A), T lymphocytes (B), B lymphocytes (C) and CD4⁺ T lymphocytes (D).

For B lymphocytes the results are different: the correlation coefficient between SP ICM and TruCount is $R = 0.96$, whereas both the slope of the linear regression line (1.01) and the bias of the Bland-Altman plot (0.3%, TruCount vs. SP ICM) indicate an excellent agreement between the SP ICM and the TruCount methods for enumerating B lymphocytes.

Similar analyses were made for comparison of the results obtained by our SP ICM method with those by DP FCM method (see Table 1). SP ICM shows a good correlation with DP FCM for enumerating leukocytes (DP FCM leukocytes count was directly obtained by hematology analyzer) ($R = 0.97$) and T lymphocytes ($R = 0.97$). However, the slopes of the linear regression lines for these populations (1.14 and 1.09, respectively) show that cell counts by our method are about 9 ~ 14 % higher than those by the DP FCM.
method. Bland-Altman plots for the same populations show a bias of $-12.0$ to $-12.9\%$ (DP FCM vs. SP ICM).

Again the results for B lymphocytes are different: the correlation coefficient is good ($R = 0.95$), but both the slope of the regression line (1.23) and the bias of the Bland-Altman plot ($-20.1\%$, DP FCM vs. SP ICM) indicate that B lymphocytes count obtained by DP FCM is about 20% lower than that by our SP ICM method.

We also performed the same comparison between the TruCount and the DP FCM methods. For enumeration of leukocytes, T lymphocytes and B lymphocytes, good correlations between TruCount and DP FCM are obtained ($R = 0.98$ to 0.99). The TruCount data are about 25% higher than those obtained from DP FCM (Slope = 1.23 to 1.26; bias = 20.5% to 22.8%, TruCount vs. DP FCM).

It is noted that in Figure 4B, when the T lymphocytes cell counts obtained by TruCount method are in excess of 2000/\mu l, most of the dots (SP ICM cell counts plotted against TruCount data) are located lower than the linear regression line. In parallel, in the corresponding Bland-Altman plot as shown in Figure 5B, when the TruCount data are higher than 2000/\mu l, all the plotted dots locate in regions above the bias line (TruCount vs. SP ICM). These results are related to the limitation of the image analysis algorithm in our system: when the cell counts are above 2000/\mu l, i.e., the cell events are above ca. 3600 per image (1 image corresponds to 1.8 \mu l of blood sample), some of the cells are too close to each other and therefore could not be counted separately; this results in the under-estimation of the cell counts.

**CD4$^+$ cell enumeration**

Different from leukocyte and its subpopulations that are uniquely defined by one specific type of cell surface antigen as we described above, CD4$^+$ cells comprises CD4$^+$ T lymphocytes and CD4$^{dim}$ monocytes. As the CD4$^+$ T lymphocytes count is essential for HIV staging, the applicability of our SP ICM system in counting CD4$^+$ cells is evaluated.

For CD4$^+$ cell enumeration, the correlation coefficients of SP ICM data with TruCount ($R = 0.83$, see Figure 4D) and with DP data ($R = 0.85$) were significantly lower than those for leukocyte and its subpopulations. SP ICM CD4$^+$ cell counts are higher than CD4$^+$ T
lymphocytes counts obtained by TruCount and by DP FCM method: the slopes of the linear regression lines are 1.42 and 1.75, respectively; and the biases are $-37.5\%$ (TruCount vs. SP ICM, see Figure 5D) and $-56.4\%$ (DP FCM vs. SP ICM), respectively.

**Immunophenotyping of CD4$^+$ Immuno-magnetically Selected Cells**

To identify the cell types other than CD4$^+$ T lymphocytes that are counted as CD4$^+$ cell by SP ICM, immunophenotyping of CD4$^+$ immuno-magnetically selected cells was performed. In this experiment, CD4 immuno-magnetically selected cells were stained with CD3 APC, CD14 PE, and CD66b FITC. Figure 6 shows the corresponding images obtained using appropriate filters in which different types of cells on the same area of the chamber upper-surface are depicted.

**FIG. 6.** Immunophenotyping of cells immuno-magnetically selected with CD4. A: 30 CD3 APC stained CD4$^+$ T lymphocytes. B: 1 CD14 PE stained monocyte (marked as M in the frame). C: 2 CD66b FITC stained granulocytes (marked as G in the frame). These images are fluorescence images from the same area on the upper-surface of the chamber. The dark particles in image B and C are red blood cells that adhered to the upper surface of the chamber.

30 T lymphocytes stained with CD3 APC, 1 monocyte stained with CD14 PE, and 2 granulocytes stained with CD66b FITC are respectively shown in Figure 6A–C. As we can see from these figures, monocytes and granulocytes can also be selected by the CD4 immuno-magnetic labeling. To get an idea of the composition of the total CD4 counts, blood specimens of 3 healthy donors were tested by immunophenotyping. The average counts of CD4$^+$ T lymphocytes, monocytes and granulocytes were respectively 808, 422
and 70 cells/µl. In these experiments the total CD4 count contained 32.5% monocytes (27.0%, 42.1% and 28.4% for each donor, respectively) and 5.4% granulocytes (5.1%, 6.0% and 5.2%, respectively).

**DISCUSSION**

The results demonstrate that the precision and the linearity of our immuno-magnetic SP ICM system are excellent.

In our study, the cell counts of leukocytes and leukocyte subpopulations (T and B lymphocytes) obtained from SP FCM (TruCount) and DP FCM (DP data) technologies showed excellent correlations ($R = 0.97$ to 0.99). The TruCount of leukocytes and leukocyte subpopulations were approximately 21% higher than the corresponding DP data (biases = 20.5% to 22.8%) (Table 1). Since DP data was obtained by multiplying the relative proportions of lymphocyte subpopulations obtained by flow cytometry with the absolute number of total leukocytes obtained from the hematology analyzer, the difference in cell counts of leukocyte subpopulations can be ascribed to the fact that the leukocytes count by TruCount is 22% higher than those by the hematology analyzer in DP FCM.

This 22% difference of leukocytes counts from TruCount and from the hematology analyzer can be explained by two possible reasons. First, we found that about 10% of the calibration beads applied in the TruCount method are aggregates of two or even three. This may induce 10% or even higher over-count in TruCount. Such over-count in SP cytometric methods using calibration beads has been reported (16). Secondly, such difference between 2 methods or between 2 labs is normal (17). For example, a collaborative study of 280 laboratories for CD4 enumeration showed a mean inter-laboratory CV of 13.7% (range 10 – 18.3%) for the SP method and 23.4% (range 14.5 – 43.7%) for the DP method (18). Consequently, the difference we observed between TruCount and the DP data is acceptable.

Our cell enumeration method shows good correlations and agreements with TruCount and DP method for counting well-defined cell populations having a unique cell membrane antigen: For CD45 leukocytes and CD3 T lymphocytes, the cell counts by our
method are in between the TruCount and the DP data, and the biases between our method and the two flow cytometric methods are less than ±13%. As these biases can be explained by the same reasons we discussed above, it demonstrates that our SP ICM system is comparable with both FCM technologies. For CD19 B lymphocytes, cell counts obtained by our method showed no significant difference with the TruCount (bias = 0.3%), but were 20.1% higher than the DP data. This discrepancy may be related to the fact that different leukocyte subpopulations have different sensitivity to red blood cell lysis buffers used in the immuno-phenotyping processes in FCM methods. (These red blood cell lysis buffers may induce membrane destruction and could result in a significantly lower leukocytes cell count. This effect is leukocyte subpopulation dependent and even individual dependent due to the patient disease and the drug treatment (19, 20)). It may also be related to the different isotype of CD19 Abs used in these two methods.

It can therefore be concluded that our method is verified for counting leukocytes, and T and B lymphocytes.

For CD4 cell enumeration, as can be expected and was proven by immuno-phenotyping, our method counts both CD4$^+$ T lymphocytes and CD4$^{dim}$ monocytes. When CD4 antibody is used to immuno-select CD4$^+$ cells, part of monocytes, that have bound enough magnetic nanoparticles to overcome the gravity and viscosity of the sample solution in the magnetic field, could be collected at the upper-surface of the chamber and counted as CD4$^+$ cells. In addition, a small amount of granulocytes, which represent the biggest leukocyte subpopulation, could non-specifically bind immuno-magnetic nanoparticles and be attracted to the upper-surface of the chamber during the magnetic selection of the CD4$^+$ cells as well.

It should be noted that for CD3 T lymphocyte and CD19 B lymphocyte enumeration, the non-specific binding of granulocytes was prevented, as the employed CD3 and CD19 cocktails contain an antibody against human Fc receptor and prevent the non-specific binding of the magnetic nanoparticles to granulocytes (from the product data-sheet). Unfortunately, this antibody is absent in the employed CD4 cocktail.
We conclude that with our immuno-magnetic SP ICM system, antibody specific cell enumeration can be successfully applied with CD45, CD3 and CD19. The immuno-magnetically selected CD4$^+$ cells contain both CD4$^+$ T lymphocytes and CD4$^{dim}$ monocytes. This clearly shows the well-known problem of monocytes interference with CD4 counts in HIV staging (21, 22). Nevertheless, immuno-phenotyping results suggest that the CD4$^+$ T lymphocytes could be enumerated in our immuno-magnetic SP ICM system by applying additional CD3 immuno-fluorescent labeling. New immuno-magnetic volumetric systems, which use extra immuno-fluorescence labeling to achieve absolute CD3$^+$CD4$^+$ T lymphocyte count, have been developed and the results in CD4$^+$ T lymphocyte enumeration will be presented in our forthcoming paper. These systems could then play a role in HIV staging in resource-constrained countries.

A major advantage of our cell enumeration system is that it is simple and easy-to-handle for sample preparation and measurement, even for less trained operators. No data interpretation is needed, which prevents subjective errors. The instrument is portable, operates on a rechargeable battery, has no moving parts, and needs no fluids. Its simplicity allows the development of affordable systems suitable for various applications. The single-unit component price of the instrument is about US$ 4,400, which is much cheaper than the cost of a flow cytometer (US$ 20,000 – 80,000) (8). Currently, the assay price is about US$ 7.5 for CD45 and CD3 enumeration, and about US$ 10.0 for CD19 enumeration. The EasySep reagents are expensive and occupy the most cost. It would be ideal to apply cheaper immuno-nanoparticle reagents. Replacing the disposable chamber (ca. US$ 1.8) with a cheaper one may further decrease the cost per test. Another potential advantage of this method is that the blood samples can be prepared and hold in chamber/magnet assembly for at least 10 hours at RT in dark, which could be useful in the field situation. These features may permit the usage of the instrument under harsh environmental conditions in resource-constrained countries. Moreover, we demonstrated that it is reliable according to the comparisons with TruCount and DP FCM technologies.
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issues: appropriate laboratory methods in the management of HIV infection.


CD4 Enumeration for HIV Monitoring

CHAPTER 4

CD4⁺ T Lymphocytes Enumeration by an Easy-to-use Single Platform Image Cytometer for HIV Monitoring in Resource-constrained Settings

ABSTRACT

Background: HIV monitoring in resource-constrained settings demands affordable and reliable CD4⁺ T lymphocytes enumeration methods. We developed a simple single platform image cytometer (SP ICM), which is a dedicated volumetric CD4⁺ T lymphocytes enumeration system that uses immuno-magnetic and immuno-fluorescent technologies. The instrument was designed to be a low-cost, yet reliable and robust one. In this paper we test the instrument and the immunochemical procedures used on blood from HIV negative and HIV positive patients.

Methods: After CD4 immuno-magnetic labeling in whole blood, CD4⁺ T lymphocytes, CD4⁺dim monocytes and some non-specifically labeled cells are magnetically attracted to an analysis surface. Combining with CD3-Phycoerythrin (PE) labeling, only CD3⁺CD4⁺ T lymphocytes are fluorescently labeled and visible in a fluorescent image of the analysis surface. The number of CD4⁺ T lymphocytes is obtained by image analysis. Alternatively, CD3 immuno-magnetic selection in combination with CD4 immuno-fluorescent labeling can also be applied for CD4⁺ T lymphocytes enumeration.

Results: The SP ICM system was compared with two single platform flow cytometer (SP FCM) methods: tetraCXP and TruCount methods. The SP ICM system has excellent precision, accuracy and linearity for CD4⁺ T lymphocytes enumeration. Good correlations were obtained between the SP ICM and the SP FCM methods for blood from HIV negative and HIV positive patients.

specimens of 44 HIV− patients, and of 63 HIV+ patients. Bland-Altman plots showed interchange-ability between the SP ICM and the SP FCM methods.

Conclusions: The immuno-labeling methods and the instrumentation are simple and easy-to-handle for less trained operators. The SP ICM system is a good candidate for CD4+ T lymphocytes enumeration in point-of-care settings of resource-constrained countries.

INTRODUCTION

AIDS is an extraordinary crisis that forms an immediate emergency and a long-term development issue. It is estimated that in 2005, 40.3 million human beings were living with HIV, of whom approximately 95% were in developing countries (1).

In recent years, the price of antiretroviral treatment per person per year has dramatically fallen from US$ 10,000 to US$ 300. By the end of 2005, more than one million persons in middle- and low-income countries had received this treatment (2). For adults, the CD4+ T lymphocytes count is essential to decide when to start the antiretroviral treatment, to monitor the effect of this treatment, and to decide when to stop opportunistic infection prophylaxis (3 - 5).

While the cost of the antiretroviral treatment has dropped dramatically, CD4+ T lymphocytes enumeration remains too expensive for many resource-constrained countries. Currently, flow cytometry (FCM) is the most widely accepted method for CD4+ T lymphocytes enumeration. However, FCM instruments are expensive and the price of an FCM assay is relatively high. Although dedicated CD4 flow cytometry systems are less expensive, the costs of the instruments and the assays are yet not affordable for resource-constrained countries. Furthermore, the operation and maintenance of an FCM requires well-trained technicians and stable electricity. Alternative methods e.g. microbead separation methods are cheap, but they are labor-intensive and may have a high operator-to-operator variations. Developing affordable, simple, easy-to-use and reliable CD4+ T lymphocytes enumeration systems is therefore urgently demanded (6 - 8).
Recently, Rodriguez et al. reported a microchip-based CD4 counting method for HIV monitoring in resource-poor settings (9). In this method, blood cells are stained with CD3 and CD4 fluorescent antibodies, captured on a membrane in a miniaturized flow cell, and then imaged through microscope optics.

We previously developed a simple single platform image cytometer (SP ICM) for absolute enumeration of white blood cells and their sub-populations in whole blood. The instrument is based on antibody-specific immuno-magnetic separation of target cells from whole blood samples, in which all the white blood cells have been labeled with a DNA fluorescent stain acridine orange (10–12). Immuno-magnetically selected cells are magnetically attracted to an analysis surface and imaged by a CCD camera. Using this method, cell types that are well defined by single type of cell surface antigen, i.e. white blood cells (CD45), T lymphocytes (CD3), and B lymphocytes (CD19), can be accurately enumerated. However, as both CD4$^+$ T lymphocytes and CD4$^{dim}$ monocytes can be selected by CD4 immuno-magnetic labeling; this procedure is not ready for the enumeration of CD4$^+$ T lymphocytes for HIV monitoring.

To overcome this limitation, we apply immuno-fluorescent labeling in addition to the immuno-magnetic labeling. Correspondingly, a dedicated SP ICM for CD4$^+$ T lymphocytes enumeration has been developed. This system identifies CD4$^+$ T lymphocytes in the fluorescent images of the analysis surface as the CD3$^+$CD4$^+$ population. The instrumentation of this system will be described in great detail elsewhere.

In this paper, the immunochemical methodology is described and the overall method is tested. Test results on HIV negative (HIV$^-$) and HIV positive (HIV$^+$) patients are presented and compared with those obtained from single platform FCM (SP FCM) methods.

MATERIALS AND METHODS

Blood Collection

Blood samples from randomly selected adult HIV$^-$ patients and adult HIV$^+$ patients were supplied by MST hospital, Enschede, The Netherlands. Blood samples from healthy donors were obtained from volunteers. All blood samples were collected in sterile
EDTA blood collection tubes (BD Biosciences, USA) by venipuncture and processed within 8 hrs after draw.

Immuno Labeling for Single Platform Image Cytometry (SP ICM)

In the SP ICM, the CD4⁺ T lymphocytes are identified as those cells that are both CD4⁺ and CD3⁺. Two different labeling schemes were respectively applied to select this population:

**Scheme a:** CD4 immuno-magnetic labeling and CD3 immuno-fluorescent labeling.

**Scheme b:** CD3 immuno-magnetic labeling and CD4 immuno-fluorescent labeling.

The appropriate amounts of immuno-magnetic labels, incubation time and magnetic separation time to be applied in SP ICM were determined by titration using blood samples from a healthy donor. CD4⁺ T lymphocytes counts of the same blood samples enumerated with SP FCM (Beckman Coulter tetraCXP 4-color method) (tetraCXP method) were used as reference. In separate titration experiments, 0.5 μl, 1 μl, 2 μl, 3 μl, 5 μl, 8 μl, 10 μl and 20 μl of immuno-fluorescent labels were applied. The appropriate amounts of immuno-fluorescent labels were determined according to the Signal-to-Noise Ratios (SNRs) of the fluorescent images and the accuracy of CD4⁺ T lymphocytes counts obtained by SP ICM. (SNR = (I_{cell} - I_{backg}) / <σ_{noise}>, where I_{cell} and I_{backg} are respectively the average fluorescence intensity of the CD4⁺ T lymphocytes and that of the background in a same image, and <σ_{noise}> is the standard deviation of the total noise present in the same image (13)). Blood specimens of 3 HIV⁺ patients, whose CD4⁺ T lymphocytes counts being ca. 200/μl, 400/μl and 600/μl (determined by SP ICM method), were used. From these titration experiments the following immuno-labeling protocols evolved:

**Scheme a:** To 100 μl of EDTA anti-coagulated whole blood, 10 μl of reagent cocktail, which contains 2 μl of 0.88 mg/ml CD4-ferrofluid (CD4FF, clone: EDU-2, isotype: mouse IgG2a-κ, Immunicon Inc., USA), 3 μl of 25 μg/ml CD3 conjugated with Phycoerythrin (CD3PE, clone: UCHT1, isotype: Mouse IgG1-κ, BD Pharmingen, USA) and 5 μl of system buffer (Immunicon Inc., USA), was added and mixed. After 15 min incubation, the sample was diluted with 290 μl of system buffer to a final volume of 400
μl. Approximately 340 μl of the sample solution was transferred into the analysis chamber. The chamber was plugged and placed into a magnet assembly (MagNest®; Immunicon Inc., USA) inside the SP ICM instrument. After 20 min magnetic separation, the sample was ready to be analyzed.

**Scheme b:** The 10 μl of reagent cocktail contains: 3 μl of 0.655 mg/ml CD3-ferrofluid (CD3FF, clone: CRIS-7, isotype: mouse IgG2a-κ, Immunicon Inc., USA), 5 μl of 12.5 μg/ml CD4 conjugated with PE (CD4PE, clone: RPA-T4, isotype: Mouse IgG1-κ, BD Pharmingen, USA) and 2 μl of system buffer. Other details are the same as those described in **scheme a**.

**CD4⁺ T Lymphocytes Enumeration by SP ICM**

During the magnetic separation period, the immuno-magnetically labeled cells were subjected to a homogenous magnetic force pointing to the positive Z-direction of the magnetic chamber (10 - 12), thus moved to the upper glass surface of the chamber (analysis surface). For excitation of Phycoerythrin (PE), 4 × 5W Light Emitting Diodes (LEDs) (470 nm, Lumileds, Luxeon, USA) are mounted symmetrically above the magnet. In front of each LED, an exciter filter (475AF50, Omega optical, USA) is placed and used to suppress the light emitted by the LED specifically in the region overlapping with the emission fluorescent signal of PE. The emission fluorescent signal is collected by a 10 × objective (NA 0.2, Lomo Optics, USA), filtered by an emission filter (595AF60, Omega optical, USA) that selects the PE fluorescence, and then focused onto an ST-402ME CCD camera (SBIG, USA). The images recorded are transferred to a single board computer (EES-3610, Evalue Technology, Taiwan) with a touch-screen monitor (B084SN03 V2, AU Optronics, Taiwan), and analyzed using a dedicated freeware image analysis algorithm that determines the number of CD4⁺ T lymphocytes per μl. For each test, three images from separate positions, each corresponding to 0.80 μl of whole blood in volume, are recorded and the counts are summed. The total volume (2.40 μl) was chosen to obtain a theoretical statistical Poisson variation of approximately 6% at 100 cells/μl and approximately 9% at 50 cells/μl (see below).
The dimensions of our SP ICM instrument are 25 cm × 25 cm × 20 cm. The system operates on 125-240 V, or on one 12 V rechargeable battery.

**CD4⁺ T Lymphocytes Enumeration by Single Platform Flow Cytometry (SP FCM)**

*TetraCXP method (Beckman Coulter, USA)*: Samples were prepared according to the manufacturer’s recommendation (14). To 100 μl of EDTA anti-coagulated whole blood, 5 μl of Cyto-stat tetraCHROME (CD45 (clone: B3821F4A, isotype: mouse IgG2b)-FITC/CD4 (clone: SFCI12T4D11, isotype: mouse IgG1)-RD1/CD8 (clone: SFCI21Thy2D3, isotype: mouse IgG1)-ECD/CD3 (clone: UCHT1, isotype: mouse IgG1)-PC5) was added and mixed. (FITC, fluorescein isothiocyanate; RD1, R-phycoerythrin; ECD, R-phycoerythrin/Texas Red tandem dye; PC5, R-phycoerythrin/cyanine5 tandem dye.) After 10 to 12 min incubation in dark at room temperature, the sample was lysed and fixed in a Multi-Q-Prep workstation. 100 μl of Flow-Count Fluospheres was added and mixed before analysis. The samples were analyzed on a Cytomics FC500 using tetraCXP software, and absolute CD3, CD4 and CD8 counts were obtained. These tests were performed in MST hospital, Enschede, The Netherlands.

*TruCount method (BD Bioscience, USA)*: TruCount™ absolute count tubes were used and blood samples were processed using a lyse-no-wash method according to the manufacturer’s recommendation (Immunocytometry System, BD Biosciences, USA) (15). Reagents used were CD3 (clone: SK7, isotype: mouse IgG1-κ) FITC/CD4 (clone: SK3, isotype: mouse IgG1-κ) PE/CD45 (clone: 2D1, isotype: mouse IgG1-κ) PerCP (TriTEST, BD Bioscience, USA). The samples were analyzed on the FACSCalibur using CELLQUEST™ software.

**Evaluation of SP ICM on CD4⁺ T Lymphocytes Enumeration**

**Precision**

To assess the precision of our SP ICM system, CD4⁺ T lymphocytes counts of five replicates from blood specimens of 20 HIV⁻ patients were measured using Scheme a. The coefficients of variation (CV = standard deviation/mean × 100%) were calculated.
Assuming that the distribution of the cell counts of the five replicate samples from each patient follows a Poisson distribution, the expected statistical Poisson variation of the cell count can be estimated according to the following equation:

\[
\text{Poisson variation} = \sqrt{\frac{2.4\mu l \times \text{cell count} / \mu l}{2.4\mu l \times \text{cell count} / \mu l}} \times 100\%
\]

where the cell count/\mu l is based on the total sampling of 2.40 \mu l of the whole blood. The CV values were compared with the Poisson variation values.

**Accuracy and linearity**

To evaluate the accuracy and linearity of our system within the clinically important range of CD4+ T lymphocytes counts (0 – 500/\mu l) (3, 16 - 18), a series of blood samples were prepared by graded dilutions of CD4+ T lymphocytes through adding CD4+ T lymphocytes depleted blood into whole blood according to the following protocol:

From a whole blood specimen of a healthy donor, a CD4 depleted blood sample was prepared using CD4FF immuno-magnetic separation method. Then the original whole blood specimen was mixed with the CD4 depleted blood sample in different ratios, resulting in a series of 5 specimens with known numbers of CD4+ T lymphocytes. The CD4+ T lymphocytes counts of the whole blood specimen, of the CD4 depleted blood sample, and of these mixed samples were enumerated by our system (scheme a). The accuracy and linearity were evaluated. As a reference, CD4+ T lymphocytes counts of the above mentioned samples were also measured by TruCount method.

**Comparison of SP ICM and SP FCM on HIV− patients**

TetraCXP method and TruCount method are frequently applied SP FCM methods for CD4+ T lymphocytes enumeration. The performance of these two methods was evaluated using blood specimens of 44 HIV− patients, and the results obtained using these two methods are compared with each other.

From the same blood specimens, the CD4+ T lymphocytes counts were also enumerated by the SP ICM methods, respectively following labeling scheme a and scheme b. The
results obtained using these two SP ICM methods are compared with each other, and also compared with those of the two SP FCM methods.

**Comparison of SP ICM and SP FCM on HIV^+ patients**

The blood specimens of 63 HIV^+ patients were tested using our SP ICM method (scheme a) and SP FCM (tetraCXP method), and the obtained CD4^+ T lymphocytes counts were compared.

**CD4^+ T lymphocytes enumeration of Immuno-Trol cells by SP ICM**

To evaluate the suitability of our SP ICM for the enumeration of CD4^+ T Lymphocytes in the stabilized cell products used in the External Quality Assurance (EQA) schemes, Immuno-Trol Cells (Beckman Coulter, USA) were separately enumerated for 10 times. The obtained CD4^+ T lymphocytes counts and the mean value were compared with the expected results (596 ± 180 CD4^+ T lymphocytes /μl) supplied by the manufacturer.

**Statistical Analysis**

Based on the CD4^+ T lymphocytes counts obtained from different methods, linear regression lines were drawn and correlation coefficients ($R$) were calculated. Bland-Altman plots (19 - 21) were used to evaluate the interchange-ability between methods. In Bland-Altman plots, the average of the CD4 counts obtained from two methods is plotted on the horizontal axis, and the difference/average% is plotted on the vertical axis. The solid line in the plot represents the bias (the average difference between the two methods), and the dashed lines in the plot illustrate the upper and lower limits of agreement (± 1.96 SD).

Percentage similarity scatter-plot and percentage histogram (22) were also used to evaluate the accuracy and precision of these methods, and the interchange-ability between methods. In percentage similarity scatter-plots, SP FCM is used as a gold standard, and the data obtained from the gold standard (vertical axis) are plotted against the percentage similarity values ($((\text{SP ICM + gold standard})/2)/\text{gold standard} \times 100$) (horizontal axis). From the plot, a mean percentage similarity, a standard deviation and a
CV (standard deviation/mean percentage similarity) can be calculated. 100% similarity means no difference between two methods. In percentage histogram, the percentage similarity data from the scatter-plot are grouped into 15 intervals, and the counts in each interval (vertical axis) were plotted against the percentage similarity (horizontal axis). A normal curve is plotted over the histogram. The spreading of the normal curve with respect to the 100% similarity reference line indicates both accuracy and precision of our method as compared to the gold standard.

RESULTS

Titration Experiments
FIG. 1. A: CD4FF titration. Number of CD4⁺ T lymphocytes/µl enumerated by ICM (scheme a) as a function of magnetic incubation time and that as a function of the amount of CD4FF applied. CD4⁺ T lymphocyte count obtained by SP FCM (tetraCXP method) was applied as a reference. Error bar is the square root of cell count. Black squares: 1 µl of CD4FF, red spheres: 2 µl of CD4FF, green up-triangles: 3 µl of CD4FF, blue down-triangles: 4 µl of CD4FF, magenta diamonds: by tetraCXP method. B: CD3PE titration. Number of CD4⁺ T lymphocytes obtained by SP ICM using scheme a, and C: Signal-to-Noise Ratios as a function of the amount of CD3PE. Black squares: the blood samples of the patient with approximately 600/µl CD4⁺ T lymphocytes, red spheres: approximately 400/µl CD4⁺ T lymphocytes, blue triangles: approximately 200/µl CD4⁺ T lymphocytes.

The appropriate amounts of immuno-magnetic reagents and magnetic separation time for selecting CD4⁺ T lymphocytes were determined by the titration experiments. Figure 1A illustrates the number of CD4⁺ T lymphocytes/µl determined using scheme a (CD4FF/CD3PE) as a function of magnetic separation time and that as a function of the amount of CD4FF applied. When 1 µl to 4 µl of CD4FF is used, the cell count reaches a plateau at 10 min of magnetic separation time. The plateau value is independent of the amount of CD4FF, and approximately equal to the CD4⁺ T lymphocytes obtained by SP FCM (tetraCXP method). Accordingly, we chose 2 µl of 0.88 mg/ml CD4FF to be applied in CD4⁺ T lymphocyte enumeration and 20 min as magnetic incubation time. These conditions ensure that all the CD4⁺ T lymphocytes could be attracted to the upper-surface of the chamber.

In order to find the appropriate amount of CD3PE used in scheme a, separate titration experiments were performed. Figure 1B and 1C illustrate the number of CD4⁺ T lymphocytes obtained by SP ICM (scheme a) and Signal-to-Noise Ratios as a function of the amount of CD3PE, respectively. It was found that the accurate CD4⁺ T lymphocytes counts could only be obtained when the amount of CD3PE is 2 µl and higher. The best SNR was obtained when using 3 µl of CD3PE. Taking the test cost into account, we chose 3 µl of CD3PE (25 µg/ml) in scheme a.
Alternatively, for scheme b (CD3FF/CD4PE), the titration experiments were performed in the same way. 3 μl of CD3FF (0.655 mg/ml) and 5 μl of CD4PE (12.5 μg/ml) were chosen.

**Precision of SP ICM**

To assess the precision of the SP ICM on CD4⁺ T lymphocytes enumeration, five replicate blood samples from 20 HIV⁻ patients (in total 100 samples) were enumerated by the SP ICM using labeling scheme a (CD4FF/CD3PE). For replicate blood samples of each patient, the calculated CV values and Poisson variations were compared.

**FIG. 2.** CV (diamonds) and Poisson variation values (squares) of CD4⁺ T lymphocytes counts from five replicates of each of the blood specimens of 20 HIV⁻ patients plotted against their mean CD4⁺ T lymphocytes counts.

Figure 2 shows the CV values and the expected Poisson variations from the five replicates of each of the 20 blood specimens, plotting against their mean CD4⁺ T lymphocytes counts. The mean CD4⁺ T lymphocytes counts range from 90/μl to 1495/μl; correspondingly, the Poisson variations range from 6.8% to 1.7%.

The measured CV values range from 7.8% to 1.8%, this range is comparable to that of the commercially available CD4⁺ T lymphocytes enumeration systems (flow cytometry and hematology analyzer) (23). Moreover, the measured CV values are close to the
expected Poisson variations; this shows that the precision of the SP ICM system is mainly determined by Poisson statistics.

**Accuracy and Linearity of SP ICM**

To evaluate the accuracy and linearity of the system, a whole blood sample and a series of diluted samples containing graded, known numbers of CD4+ T lymphocytes were prepared. Both the original undiluted sample and the diluted preparations were checked in parallel with the “gold standard” BD TruCount method (15) and the SP ICM (scheme a). In figure 3A, the CD4+ T lymphocytes counts measured by SP ICM were plotted as a function of the percentage of the whole blood in these samples (0% for the CD4 depleted sample, and 100% for the original whole blood sample). A linear relation (R = 0.998) is found, demonstrating the linearity of our system.

A

![Graph A](image)

\[ y = 5.57x + 29 \]
\[ R = 0.998 \]

B

![Graph B](image)

\[ y = 0.98x \]
\[ R = 0.997 \]
FIG. 3. Linearity and accuracy of the SP ICM system for CD4+ T lymphocytes enumeration. A: Observed SP ICM CD4+ T lymphocytes counts of whole blood sample of a healthy donor, its CD4 depleted blood sample, and a series of their mixtures vs. the percentage of the whole blood in these samples (for the original whole blood sample, 100%; for the CD4 depleted blood sample, 0%). B: Corrected SP ICM CD4+ T lymphocytes counts vs. CD4+ T lymphocytes counts obtained using SP FCM (TruCount method).

In figure 3A, an offset of 29 cells/μl was found for CD4 depleted samples. These cells are probably the CD4+ T lymphocytes that are left over in the CD4 depleted blood; they carry magnetic labels as a result of the depletion procedure, but have not been removed and therefore could be detected by the SP ICM. In SP FCM analysis, such an offset is not present. This result is logic: since the CD4 receptors on these remaining CD4+ T lymphocytes in the CD4 depleted blood have been covered with the immuno-magnetic labels, these cells can not be immuno-fluorescently labeled and recognized in SP FCM analysis.

Considering the presence of these remaining CD4+ T lymphocytes, the cell counts of each sample obtained by SP ICM can be ‘corrected’ by taking out the contribution of these remaining cells. Such ‘corrected’ SP ICM counts are compared with the cell counts obtained from SP FCM, as shown in figure 3B. From the fact that the slope of the curve is close to 1.0 (0.98) and R is 0.997, we conclude that the SP ICM method and the SP FCM method give comparable and accurate results.

Comparing SP ICM with SP FCM on HIV− Patients

First, the two ‘gold standard’ SP FCM methods, Tetra CXP method and TruCount, were compared with each other by enumerating CD4+ T lymphocytes for 44 HIV− patients (CD4+ T lymphocytes counts: 128 - 1958/μl, obtained by tetraCXP method). Figure 4 shows the linear regression plot (Fig. 4A) and the Bland-Altman plot (Fig. 4B) of this comparison. From figure 4A a correlation coefficient (R) of 0.99 and a slope of 0.91 were determined, with the tetraCXP data being about 9% lower than the TruCount
data. Figure 4B shows a bias of −8.0% and limits of agreement (mean value ± 1.96 Standard Deviation) of −19.1% to 3.0% (tetraCXP data vs. TruCount). These data show that the two gold standards yield comparable, but slightly different values.

**FIG. 4.** Linear regression plot (A) and Bland-Altman plot (B) comparing CD4+ T lymphocytes enumerations using tetraCXP and TruCount methods. Blood specimens of 44 HIV− patients were tested.

Then we compared the SP ICM results obtained respectively using the labeling scheme a and scheme b for the same 44 HIV− patients. The results are shown in figure 5. From figure 5A a correlation coefficient of 0.99 and a slope of 0.95 were calculated; the Bland-Altman plot in figure 5B shows a bias of −4.6% and limits of agreement of −19.9% to 10.8% (scheme b vs. scheme a). These results illustrate that the two labeling methods
agree well with each other, with scheme b giving results about 5% lower than those from scheme a. This difference is smaller than that we found between the two ‘gold standards’.

The SP ICM results obtained from labeling scheme a and scheme b were respectively compared with the results obtained from the two ‘gold standard’ SP FCM methods. The results are shown in figure 6 (comparing with TruCount) and figure 7 (comparing with tetraCXP).

FIG. 5. Linear regression plot (A) and Bland-Altman plot (B) comparing CD4$^+$ T lymphocytes enumerations by SP ICM using labeling scheme b and scheme a. Blood specimens of 44 HIV$^-$ patients were tested.
FIG. 6. Linear regression plots and Bland-Altman plots comparing CD4\(^+\) T lymphocytes enumerations by SP ICM using respectively scheme \textit{a} (A and B) and scheme \textit{b} (C and D) vs. the enumeration by TruCount SP FCM. Blood specimens of 44 HIV\(^-\) patients were tested.

The CD4\(^+\) T lymphocytes counts obtained by both SP ICM methods are comparable to and slightly less than those obtained by TruCount method. In case that labeling scheme \textit{a} was applied, the correlation coefficient \(R\) is 0.98, the slope is 0.95, and the bias is \(-0.8\%\), with limits of agreement of \(-15.2\%\) to 13.7 \% (Fig. 6A and 6B). In case that labeling scheme \textit{b} was applied, \(R\) is 0.97, the slope is 0.90, and the bias is \(-5.3\%\), with limits of agreement of \(-27.3\%\) to 16.6 \% (Fig. 6C and 6D).
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FIG. 7. Linear regression plots and Bland-Altman plots comparing CD4\(^+\) T lymphocytes enumerations by SP ICM using respectively scheme \(a\) (A and B) and scheme \(b\) (C and D) vs. the enumeration by tetraCXP SP FCM. Blood specimens of 44 HIV\(^-\) patients were tested.

Figure 7 shows the comparison of the two SP ICM methods with the tetraCXP method. The CD4\(^+\) T lymphocytes counts obtained by both the SP ICM methods are comparable to and slightly more than those obtained by the tetraCXP method. In case of labeling scheme \(a\), \(R\) is 0.99, the slope is 1.04, and the bias is 7.2\% with limits of agreement from −6.9\% to 21.3\%, as illustrated in Fig. 7A and 7B. In case of labeling scheme \(b\), \(R\) is 0.98, the slope is 0.99, and the bias is 2.7\% with limits of agreement from −19.4\% to 24.8\% (Fig. 7C and 7D).

These results indicate that the SP ICM methods following both immuno-labeling schemes agree well with the two SP FCM methods. The bias values are largest between
the two gold standards; and the SP ICM data are in between of those obtained from
tetraCXP method and from TruCount method. The SP ICM methods are interchange-able
with SP FCM methods for HIV− patients.

When comparing the data obtained from scheme a and from scheme b in SP ICM
methods (using the ‘gold standards’ as reference), it appears that the range of limits of
agreement between the SP ICM and the SP FCM is smaller when scheme a (see Fig. 6B
and 7B) is used, as compared to the case using scheme b (see Fig. 6D and 7D).

From Figure 4B, 6B, and 6D, it is noticed that for blood samples with CD4+ T
lymphocytes counts above ~ 1400/μl, the cell counts from both tetraCXP method and our
two SP ICM methods are less than those from TruCount.

Comparing SP ICM with SP FCM on HIV+ Patients

In view of the abnormalities of the immune system in HIV infected patients (23), we
evaluated the SP ICM method (scheme a) separately by enumerating the CD4+ T
lymphocytes in blood samples of 63 adult HIV+ patients. SP FCM tetraCXP method
was also used for the same blood samples (from tetraCXP method, the CD4+ T
lymphocytes counts of these patients range between 8 and 1134/μl). The accuracy of the
SP ICM method and the interchange-ability of the SP ICM method with the SP FCM
were assessed (see Fig. 8). From figure 8A it is clear that a good correlation between the
two methods was obtained, the correlation coefficient $R$ is 0.97, and the slope of the
regression line is 0.98. The Bland-Altman plot (Fig. 8B) shows a small bias of only
1.4% with the limits of agreement (± 1.96 SD) ranging from −29.4% to 32.2% (SP ICM
vs. TetraCXP). These results demonstrate the interchange-ability between the SP ICM
and the tetraCXP method for testing blood samples from HIV+ patients.

The percentage similarity scatter-plot (Fig. 8C) and the histogram (Fig. 8D) show an
excellent agreement as well. In figure 8C, tetraCXP data are used as the gold standard,
and the data obtained from the gold standard are plotted against percentage similarity
values ($\frac{(\text{SP ICM + gold standard})}{2}/\text{gold standard} \times 100$). A mean percentage
similarity of 101.47% is calculated, with a standard deviation of 10.15% and a CV of
10.00%. If one outlier with extremely low CD4+ T lymphocytes count (tetraCXP: 8/μl,
SP ICM: 18/µl, see Figure 8 A-C) is excluded, the results are 100.49% for mean percentage similarity, 6.53% for standard deviation, and 6.50% for CV. Similarly, the normal curve in figure 8D spreads around the 100% similarity reference line, peaking at approximately 101.5%. These results confirm the accuracy and precision of the SP ICM method for CD4+ T lymphocytes enumeration of HIV+ patients.

FIG. 8. Linear regression plot (A), Bland-Altman plot (B), percentage similarity scatter-plot (C), and percentage similarity histogram (D) comparing CD4+ T lymphocytes enumeration by SP ICM using labeling scheme a with that by tetraCXP SP FCM (the gold standard). Blood specimens of 63 HIV+ patients were tested. An outlier is indicated with a circle in each figure. In (D), a normal curve is plotted.

CD4+ T Lymphocytes Enumeration of Immuno-Trol Cells by SP ICM

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It is required by WHO that the new CD4 counting devices should work with specimens used in the External Quality Assurance (EQA) schemes. Immuno-Trol Cells are stabilized blood cells product which is used for QASI (Quality Assurance Systems International, an international program for quality assessment and standardization for T lymphocyte subset enumeration) (24). Therefore, our SP ICM system was used to count CD4+ T lymphocytes in Immuno-Trol Cells product. The mean CD4+ T lymphocytes count of 10 measurements was 585/μl, which is very close to the expected count (596 ± 180 CD4+ T lymphocytes/μl) as supplied by the manufacturer. Furthermore, the obtained CD4+ T lymphocytes counts ranged from 507 to 680/μl, this range is much smaller than that supplied by the manufacturer (from 416 to 776 CD4+ T lymphocytes/μl).

**DISCUSSION**

The results demonstrate that the SP ICM method for enumerating CD4+ T lymphocytes works well on blood samples of healthy donors, HIV− patients, HIV+ patients, and the stabilized blood cells product (Immuno-Trol cells).

Our method is based on the combination of immuno-magnetic separation and immuno-fluorescent labeling of target cells. The counted cells are CD3+CD4+ T lymphocytes, while CD4+ monocytes are excluded in this way. The fact that the selected cells are positive for two antibodies brings in the options of two different labeling schemes, namely CD4FF/CD3PE (scheme a) and CD3FF/CD4PE (scheme b). Both schemes give exchangeable results with each other, and more importantly, with the two SP FCM methods that are frequently used as gold standard. The precision, the accuracy and the linearity of the SP ICM are excellent in the clinically important range of CD4+ T lymphocytes counts (0 ~ 500/μl) for monitoring HIV induced immunodeficiency.

Our system directly outputs the values of interest: A small computer controls the measurement cycle and supports image analysis program. Additionally, the sample measurement is an automatically controlled process, and the CD4+ T lymphocytes count is automatically displayed within 1 min after insertion of the analysis chamber into the instrument. The CD4+ T lymphocytes counts of the patients are available in about 40 min.
after blood draw. Accordingly, the clinician can make a decision about treatment or non-treatment of the patients during their first visit. This eliminates delays, costs and other problems that may be generated by shipping the blood samples to central hospital labs. One well-trained operator can carry out about 50 tests with one instrument and several MagNest® in 8 hrs.

Our SP ICM instrument applies well-known principles of fluorescent microscopy and cheap components where possible. For illumination, cheap and long lifetime LEDs are used. The SP ICM is simple and easy-to-handle, even for less trained operators. No data interpretation is needed, which prevents subjective errors. Acquiring pipetting skill for the 3 pipetting steps in sample preparation is the most essential part in training the operators. Moreover, the system is portable, robust, battery-operated, and suitable for point-of-care applications. It can be operated immediately after arrival at site, and can even be operated on a car battery. Due to the simplicity and the fixed position of the optical components, no alignment calibration is required. All these aspects are especially advantageous for usage in developing countries.

Although the system operates well, in some aspects further improvements would be ideal. In the first place this concerns a further reduction of the price. The single-unit component price of the instrument is about US$ 3,000, much cheaper than the cost of a flow cytometer. A considerable percentage of the assay price (ca. US$ 3) comes from the disposable chamber (ca. US$ 1.8), suggesting the necessity to replace this chamber with a cheaper one. A different concern is the stabilization and packaging of the reagents for applications under tropical conditions. Pre-packaging of the reagents in a cheaper and smaller disposable test chamber would further decrease the manual steps and the amount of the employed reagents.

It is found that for some HIV+ patients when their blood specimens were aged over 24 hrs, our CD4+ T lymphocytes enumeration system (scheme a) gives over-counts, whereas for blood specimens of healthy donors, the CD4+ T lymphocytes could reliably be enumerated at up to 72 hrs after blood draw. This is likely caused by the fact that for aged blood samples of some HIV+ patients, a part of the CD3+CD8+ T lymphocytes are non-specifically (magnetically) selected, and counted as CD4+ T lymphocytes since they
are labeled with CD3PE. Such non-specific selection probably arises due to the abnormalities of the immune system in HIV infected patients (25).

It is important to carefully select the source of immuno-magnetic nanoparticles because unexpected effects may occur. In our study, EasySep nanoparticles (StemCell Technologies, Canada) and ferrofluid (FF) nanoparticles (Immunicon Inc., USA) have been tested. Both nanoparticles are iron-oxide particles with an average size of 170 nm. The EasySep particles give better image (less clustering of particles). However, using EasySep CD4 immuno-magnetic nanoparticles in combination with CD3PE labels, we found 2 outliers amongst 76 HIV+ patients (results not shown). The CD4+ T lymphocytes counts of these two patients obtained from SP ICM method were 3-5 times higher than those obtained from SP FCM method. These over-counts were related to a particular batch of the EasySep immuno-magnetic nanoparticles and to particular patients. These artifacts were not encountered when the same blood samples of these two patients were tested using FF nanoparticles. Consequently, we selected FF particles for use in our system for HIV+ patients. We could not find the reason for this phenomenon. Whether it is related to the over-count occurring upon aging blood of some HIV+ patients as we described above is unknown.

Determination of CD4/CD8 ratios is important for pediatric HIV diagnosis and monitoring (26, 27). Currently, a system that simultaneously counts CD4+ and CD8+ T lymphocytes applying the same methodology is under development. The SP ICM system described in this article and the new CD4+ and CD8+ enumeration system will be tested in resource-constrained countries.

In conclusion, the SP ICM system is a good candidate for CD4+ T lymphocytes enumeration in point-of-care settings of resource-constrained countries.

ACKNOWLEDGEMENTS

This study is financially supported by STW, the Dutch Technology Foundation (project TGT. 6146). We thank M.D. Ph.D. I. Vermes and M.D. Ph.D. C.H.H. ten Napel from MST hospital in Enschede for kindly supplying fresh blood samples and FCM data. We acknowledge all patients and healthy donors whose blood was tested in this study.
REFERENCES


CHAPTER 5

CD4 and CD8 Enumeration by an Easy-to-use Single Platform Image Cytometer for Point-of-care Pediatric HIV Monitoring in Resource-constrained Settings

ABSTRACT

Background: HIV monitoring of children in resource-constrained settings demands affordable and reliable CD4⁺ and CD8⁺ T lymphocytes enumeration methods. We developed a volumetric single platform image cytometer (SP ICM), dedicated to count CD4⁺ and CD8⁺ T lymphocytes for pediatric HIV monitoring. This system uses immuno-magnetic selection and immuno-fluorescent labeling technologies. The instrument was designed to be low-cost, yet reliable, easy-to-use and robust.

Methods: Only one-step immuno-labeling of whole blood with CD3-magnetic nanoparticles, CD4-Phycoerythrin (PE) and CD8-Peridinin-Chlorophyll-Protein Complex (PerCP) is required. Then CD3 cells are immuno-magnetically attracted to an analysis surface, where fluorescence images of CD4⁺ and CD8⁺ T lymphocytes are recorded and analyzed, respectively. In this paper we compared CD4, CD8 count and CD4/CD8 ratio by the SP ICM with those from a SP flow cytometer (FCM) tetraCXP method on blood samples from 50 HIV⁻, 50 HIV⁺ adults and 45 HIV⁻ children.

Results: Good correlations were obtained for all the measurements (R: 0.94 ~ 0.99). For HIV⁻ adults and HIV⁻ children, the slopes of CD4 and CD8 count comparisons varied from 1.08 to 0.94. For HIV⁺ adults, these numbers were somewhat lower, varying from 0.99 to 0.81. The CD8 under-count may be partly caused by a small percentage of

A part of this chapter is to be submitted for publication. Li X, Breukers C, Ymeti A, Lunter B, Terstappen LWMM, Greve J. CD4 and CD8 enumeration for HIV monitoring in resource-constrained settings.
CD8^{dim} T lymphocytes that were not detected by the SP ICM or not counted due to the interference of the cross-talked PE image of CD4^{+} T lymphocytes.

**Conclusions:** The SP ICM system is a good candidate for pediatric HIV monitoring in point-of-care settings of resource-constrained countries.

**INTRODUCTION**

Every day about 1,500 children under 15 years old are infected with HIV; more than 90% of them live in the developing countries, and most are infected by mother-to-child transmission. HIV-infected infants frequently present clinical symptoms in the first year of life. One year after birth approximately one third of the infected infants have died, and after 2 years about half of them have died. Therefore, HIV diagnosis and monitoring of infants and children in developing countries is of great importance (1).

**FIG. 1.** The CD4, CD8 counts (A) and CD4/CD8 ratios (B) as a function of age from healthy neonatal to healthy adult. For each age group, the median values and the 5th and 95th percentiles are shown. The red box shows the age range of the 45 HIV^- children tested in this study. The star indicates the mean value of their ages. The red arrow bar
shows the CD4/CD8 ratio of 1 as the indicator of HIV infection for children younger than 2 years old.

For HIV infected adults, the CD4 T lymphocytes counts are essential to decide when to start the antiretroviral therapy (ART) (< 350 cells/μl), to monitor the effect of this treatment, and to decide when to stop opportunistic infection prophylaxis (2-4). For children the reference ranges of CD4 and CD8 values as percentages of total lymphocytes, or as absolute numbers, and the reference ranges for the CD4/CD8 ratios are age dependent and different from those of adults (5-7). Figure 1 shows the CD4, CD8 counts (A) and CD4/CD8 ratios (B) as a function of the age from healthy neonatal to healthy adult (5-7). The number of CD4 T lymphocytes increases 1.5-fold immediately after birth and gradually decreases 3 fold from 2 years to adults. While CD8 T lymphocytes remain stable from birth up to 2 years of age, followed by a gradual 3-fold decrease toward adult levels. Therefore, the criteria for HIV staging of children are also different from those for adults.

The World Health Organization (WHO) recommends initiation of highly active antiretroviral therapy (HAART) for HIV positive infants under the age of 18 months, who have WHO Pediatric Stage III disease and CD4 percentage < 20%. If the CD4 percentage is not available, absolute CD4 count thresholds may be used for indication of severe HIV immunodeficiency and may be used to initiate ART (i.e. < 1500 cells/μl for infants aged ≤ 11 months, < 750 cells/μl for children aged 12-35 months, or < 350 cells/μl for children aged 36-59 months). For children aged 5 years and above the same cut-off values as in adults, i.e. < 200 cells/μl, can be used (1).

In resource-constrained countries, the standard virological tests (HIV DNA, HIV RNA, or p24 antigen), for definitive diagnosis of HIV infection for children younger than 18 months, are not available (8). The WHO encourages approaches to presumptive clinical diagnosis of HIV infection for children younger than 18 months, including studies on CD4 percentage or CD4/CD8 ratio combined with clinical signs and symptoms (1). Zijenah et al. have shown that the CD4/CD8 ratio appeared a potential tool in diagnosis of HIV infection for infants younger than 2 years old: the CD4:CD8 ratio above 1
corresponds to HIV negative, while the ratio below 1 corresponds to HIV positive. The CD4/CD8 ratio had a sensitivity of > 98% for diagnosis of HIV-1 infection and a specificity of > 98%. When necessary, the CD4/CD8 ratio may be used with caution to diagnose HIV infection (9). Therefore, a simple test that can provide CD4 and CD8 counts, and a CD4/CD8 ratio, would be very useful for presumptive HIV diagnosis and monitoring of infants younger than 2 years old in resource poor countries.

Currently, flow cytometry (FCM) is the most widely accepted method for CD4+ T and CD8+ T lymphocytes enumeration. However, FCM instruments are expensive (US$ 20,000 ~ 125,000) and the price of an FCM assay (US$ 5 ~ 50) is relatively high. Furthermore, the operation and maintenance of an FCM requires well-trained technicians and stable electricity. Affordable, simple, easy-to-use and reliable systems for CD4+ and CD8+ T lymphocytes enumeration are therefore urgently demanded (10 - 12).

Previously we developed the StarCount 2.0, a CD4+ T lymphocytes enumeration system applying immuno-magnetic selection and immuno-fluorescent labeling technologies dedicated to monitor adult HIV infection (13, 14). The instrument, a simple single platform image cytometer (SP ICM), was designed to be a low-cost (components cost US$ 3,000), compact, yet reliable, easy-to-use and robust one. It is computer-controlled, and can operate on a 12 V rechargeable battery. Only one reagents incubation step is needed in sample preparation, which makes training very easy for the new users.

Further development of the SP ICM leads to the StarCount 2.1 system (14) used for CD4+ T and CD8+ T lymphocytes enumeration and CD4/CD8 ratio determination. In this system, CD3 immuno-magnetic selection in combination with CD4 and CD8 immuno-fluorescent labeling are applied for CD4+ T and CD8+ T lymphocytes enumeration. First CD3 cells in whole blood are immuno-magnetically attracted to an analysis surface. Then CD4+ T lymphocytes and CD8+ T lymphocytes can be counted in fluorescence images as they are labeled with CD4-Phycoerythrin (PE) and CD8-Peridinin-Chlorophyll-Protein Complex (PerCP), respectively. With this information the CD4/CD8 ratio can then be determined.
In this paper we evaluated the system for its ability to count CD4⁺ T and CD8⁺ T lymphocytes and determine the CD4/CD8 ratio. The results were compared with those obtained by the SP FCM tetraCXP method. Blood specimens of 50 HIV⁻ adults, 50 HIV⁺ adults and 45 HIV⁻ children (0 – 10 years old, 4.6 ± 3.4 (mean ± SD), as shown in the Figure 1) were tested. Because there are very few HIV⁺ children in the Netherlands, the field tests on HIV⁺ children will be carried out later in other countries (see chapter 7).

MATERIALS AND METHODS

Blood Collection

Blood samples from randomly selected patients were supplied by MST hospital, Enschede, The Netherlands. The patients consisted of 50 HIV⁻ adults, 50 HIV⁺ adults and 45 HIV⁻ children (0 – 10 years old, 4.6 ± 3.4 (mean ± SD)). All blood samples were collected in sterile K₃EDTA blood collection tubes (BD Biosciences, USA) by venipuncture or heel-prick (for infants) and processed within 8 hrs after draw.

CD4⁺ T and CD8⁺ T Lymphocytes Enumeration by Single Platform Flow Cytometry (SP FCM)

TetraCXP method

Samples were prepared according to the manufacturer’s recommendation (Beckman Coulter, USA) (15). To 100 μl EDTA anti-coagulated whole blood, 5 μl of Cyto-stat tetraCHROME (CD45 (clone: B3821F4A, isotype: mouse IgG2b)-FITC/CD4 (clone: SFI21T4D11, isotype: mouse IgG1)-RD1/CD8 (clone: SFI21Thy2D3, isotype: mouse IgG1)-ECD/CD3 (clone: UCHT1, isotype: mouse IgG1)-PC5) was added and mixed. (FITC, fluorescein isothiocyanate; RD1, R-phycoerythrin; ECD, R-phycoerythrin/Texas Red tandem dye; PC5, R-phycoerythrin/cyanine5 tandem dye.) For samples of infants younger than 2 years old, 25 μl whole blood instead of 100 μl was used. After 10 to 12 min incubation in the dark at room temperature, the sample was lysed and fixed in a Multi-Q-Prep workstation. 100 μl of Flow-Count Fluorospheres was added and the sample was mixed before analysis. The samples were analyzed on a Cytomics FC500
using tetraCXP software, and absolute CD3, CD4 and CD8 counts were obtained. These tests were performed in the MST hospital, Enschede, The Netherlands.

**CD4⁺ T and CD8⁺ T Lymphocytes Enumeration by SP ICM**

One-step immuno labeling

To 100 μl (25 μl was used for blood samples from children) EDTA anti-coagulated whole blood, 10 μl of reagents cocktail, which contains 3 μl of 0.655 mg/ml CD3-ferrofluid (CD3FF, clone: CRIS-7, isotype: mouse IgG2a-κ, Immunicon Inc., USA), 4 μl of 12.5 μg/ml CD4PE (clone: RPA-T4, isotype: Mouse IgG1-κ, BD Pharmingen, USA) and 3 μl of 6.25 μg/ml CD8PerCP (clone: SK1, isotype: Mouse IgG1-κ, BD BioScience, USA) was added and mixed. After 15 min incubation, the sample was diluted with system buffer to a final volume of 400 μl. Approximately 340 μl of the sample solution was transferred into the analysis chamber. The chamber was plugged and placed into a magnet assembly (MagNest®; Immunicon Inc., USA). After 20 min magnetic separation, the sample was ready to be analyzed.

**Instrument**

During the magnetic separation period, the CD3 immuno-magnetically labeled cells are subjected to a homogeneous magnetic force pointing in the positive Z-direction of the magnetic chamber (16 - 18). The cells then move to the upper glass surface of the chamber (analysis surface). For excitation of PE and PerCP, 2 × 3 W Light Emitting Diodes (LEDs) (496 nm, Lumileds, Laxeon, USA) are mounted symmetrically above the magnet. In front of each LED, a 550 nm short pass filter (Omega Optical, USA) is placed to suppress the light emitted by the LED in the region overlapping with the emission fluorescent signal of PE and PerCP. The emission fluorescent signal is collected by a 10 × objective (NA 0.2, Lomo Optics, USA), and filtered by emission filters mounted on a filter slider. Two emission filters were used: one (595AF60, Omega Optical, USA) selects the PE fluorescence; the other (695AF55, Omega Optical, USA) selects PerCP fluorescence. The filtered fluorescence is focused onto an ST-402ME CCD camera (SBIG, USA). The images recorded are transferred to a single board computer (665 MHz...
CD4 and CD8 Enumeration for Pediatric HIV Monitoring

The dimensions of the SP ICM instrument are 25 cm × 25 cm × 20 cm. The system operates on 125 - 240 V, or on one 12 V rechargeable battery. With a fully charged 12 V, 7 Ah lead-acid battery, the SP ICM instrument can run for approximately 2 hours and analyze about 15 samples for both CD4 and CD8 enumeration. A small 30 Ah car battery is able to support the instrument for 8 hours.

Image analysis algorithms

The algorithm is written in ImageJ (NIH, Maryland, USA), a public-domain Java image processing software package (19). The basic algorithm consists of the following steps: 1. Background subtraction: Corrects for non-uniform illumination, variation in the sensitivity of the CCD pixels etc., using a rolling ball averaging. 2. Thresholding: Transforms the grey-scale image to a binary image. All the objects (cells) that have higher intensity than a pre-set threshold are assigned as 1, and the rest of the objects that have lower intensity (background) are assigned as 0. 3. Watershed: Separates cells in the image and marks the boundaries of each single object (cell). The procedure first determines the centre of the separate cells (peak intensities), then dilates until the edge of a cell or the edge of an adjacent cell is reached. 4. Counting: Enumerates all objects (cells) assigned as 1. 5. Separation: An additional step that first estimates the average area of a cell in the image and then divides the total area of overlapping cells by this average cell area. This step is used to correct the cell count for events that are lying too close to each other to be separated by the watersheding procedure. 6. Fill error: The above procedures cannot separate cells with a large or complete overlap. This creates a counting error.
error (loss) that increases with the number of cells in the CCD image. To estimate this counting error, we simulated images with a known number of randomly deposited cells, and applied the image analysis algorithm to these artificial images. The real number of cells in the image was plotted against the counted number of cells (Figure 2). A second order polynomial regression equation could be fitted to the data. In the measurements we use this formula to correct the number of cells counted. The correction is < 1 % at 100 cells/µL, < 2.5 % at 500 cells/µL, and < 7.5% at 1000 cells/µL.

**FIG. 2.** Simulation of the counting error (under-count) appearing by applying the Watersheding to separate overlapping cells. The curve of the number of cells in the images (0 ~ 2326 cells/image or 0 ~ 2004 cells/µL) against the counted cell number was fitted by a polynomial regression.

**Setting the threshold**

Setting a correct value for the threshold is one of the most difficult points in the image analysis. Several routines can be followed. A well-trained operator may first set the threshold manually, and accordingly the single board computer counts the number of events in the image. The procedure is then repeated until the number counted is invariant under small variations of the threshold. This ‘trained operator’ manual threshold method has been tested many times in the last two years by comparing the results with FCM data.
This method works well, provided an experienced operator sets the threshold values for the different images, and judges the accuracy of counting by checking the images of those cells that have been counted. In the end the analyzed image is shown on the touch-screen with the counted cells encircled in red. Analysis of one image for one threshold level setting takes the single board computer (665 MHz processor with 256 MB RAM) about 20 ~ 30 seconds.

Since the goal is to have a fully automatic counting in the SP ICM, all images were also saved and then analyzed extensively using a PC to test two new developed automatic image analysis algorithms.

First, a dynamic threshold method was developed that mimics the trained operator manual threshold method. An iterative algorithm counts the number of cells per image at different threshold level settings, starting at background level. Counting is repeated many times with stepwise increased threshold settings. Iteration is stopped when the CV of 5 consecutive counts is smaller than a preset value, e.g. 5 or 10%. The threshold is taken as the value used when the CV for the first time is smaller than this percentage. However, the dynamic threshold method appeared to be very time consuming, due to the repetitive calculation steps needed. For images with cell counts from ~ 50/μl to ~ 1200/μl, this method takes ~ 3 min to ~ 10 min to determine the threshold by using the single board computer.

The second method that was developed and tested is the maximum entropy threshold method (20). In this method, the image gray level histogram is divided into two probability distributions, one representing the objects and the other representing the background. The optimal threshold is defined such that the sum of the entropies of these probability distributions is maximized. It takes only ~ 20 to ~ 30 seconds to analyze images with cell counts from ~ 50/μl to ~ 1200/μl by using the single board computer.

The dynamic threshold method and the maximum entropy threshold method were applied to analyze the CD4PE images of all samples in this test by using a PC, and the results were compared with the CD4 counts obtained by the FCM and by the trained operator manual threshold method.
To investigate whether the SP ICM is able to detect the weak PerCP fluorescence of CD8dim T lymphocytes, magnetic beads with the same diameter as cells were coated with different amounts of PerCP molecules (by biotin-streptavidin conjugation) to mimic cells with different PerCP intensities. Biotinylation of paramagnetic beads (Compel magnetic, COOH modified, 8 μm; Bangs Laboratories, Inc., USA) was performed by Immunicon Inc. The biotinylated paramagnetic beads were incubated with different amounts of streptavidin-PerCP (SAV-PerCP, BD, USA). To 30 μl biotinylated paramagnetic beads, 3 μl, 5 μl, 5.5 μl, and 6 μl of SAV-PerCP were added, respectively. After 30 min incubation at RT in the dark, the PerCP-magnetic beads were washed twice with PBS (0.1% BSA) under magnetic separation to remove the free SAV-PerCP. The PerCP-magnetic beads were re-suspended in PBS (0.1% BSA). Four groups of PerCP-magnetic beads with different fluorescent intensities were obtained and their fluorescent GeoMean Intensities (GI = \( \prod a_i \) ) were quantified by FCM (FACSCalibur, BD, USA) and by the SP ICM. In the FCM, four groups of PerCP-magnetic beads were mixed with a blood sample stained with CD8PerCP. The GIs of the PerCP-magnetic beads were compared with those of CD8bright and CD8dim T lymphocytes in the same sample. In the SP ICM, the four groups of PerCP-magnetic beads were respectively mixed with a blood sample stained with CD4PE and CD8PerCP. CD4PE and CD8PerCP were added to introduce the same background as in a real blood sample for CD8 enumeration. In this case CD4PE was also added because of the crosstalk of the PE emission through the PerCP filter (see below).

Then a known number of two groups of PerCP beads, with different PerCP intensities, were added to one blood sample to mimic the CD8PerCP labeled CD8bright and CD8dim T lymphocytes. CD4PE and CD8PerCP were also added. The images were recorded, and analyzed to check whether beads with two different intensities could be detected simultaneously in one image, or not. The blood sample was taken from a randomly selected healthy donor.
PE fluorescence cross-talk in PerCP image

Figure 3 illustrates the spectra of LED (only central wavelength), excitation and emission of PE and PerCP, the 550nm short pass excitation filter, the 595AF60 band pass filter and the 695AF55 band pass filter (21). It is clear that the long PE emission tail (estimated at ~ 2%) penetrates through the PerCP filter. This cross-talk of the CD4 PE cells shows dimly in the CD8PerCP fluorescence image. This is demonstrated in Figure 4A-C. A CD4PE image, a CD8PerCP image, and the color merge of these two images are shown. The CD4PE cells light up dimly in the PerCP image. The CD8PerCP dim cells have approximately the same intensity as the cross-talk of the CD4PE cells. CD8PerCP dim cells shown in the image are CD8dim T lymphocytes with (for dim cells) relatively high intensity, known by the previous PerCP beads experiment. Cells with lower intensities will be missed if the threshold in the counting algorithm is set too high. If this is the case the SP ICM system will only count the CD8bright T lymphocytes yielding a CD8 under-count by the SP ICM when compared to the FCM.

FIG. 3. Spectra of LED (only the central wavelength), excitation and emission of PE and PerCP, 550nm short pass filter as excitation filter, 595AF60 band pass filter for PE fluorescence and 695AF55 band pass filter for PerCP fluorescence. The long PE emission tail penetrates through PerCP filter.
FIG. 4. A: CD4PE image. B: CD8PerCP image. C: Color merge of CD4PE (green) and CD8PerCP (red) images. Cells in yellow circle indicate CD4PE cells (in A), which are shown dimly in the CD8PerCP image (in B). Cells in blank circle indicate CD8^{dim+}T cells (in B and C). (color figures on Page 212)

Statistical Analysis
Based on the CD4⁺ T, CD8⁺ T lymphocytes counts and CD4/CD8 ratio obtained from different methods, linear regression lines were drawn and correlation coefficients (\( R \)) were calculated. Bland-Altman plots (22, 23) were used to evaluate the interchange-ability between methods. In Bland-Altman plots, the average of the CD4 or CD8 counts or CD4/CD8 ratio obtained from two methods is plotted on the horizontal axis, and the difference/average (%) is plotted on the vertical axis. The solid line in the plot represents the bias (the average difference between the two methods), and the dashed lines in the plot illustrate the upper and lower limits of agreement (bias ± 1.96 SD). The 95% confidence interval (CI) of the bias is also shown.

RESULTS

SP ICM

Sensitivity of SP ICM to detect CD8⁺dim T lymphocytes

FIG. 5. Automatic gating in the scatter plot of CD8-ECD vs. CD3-PC5 from a SP FCM for CD8⁺ T lymphocytes count (A), and the manual gating for CD8⁺bright T lymphocytes count in the histogram of CD8-ECD from CD3⁺ cells (B).

It is well known that CD8⁺dim T Lymphocytes may have a fluorescent intensity that is up to 50 times lower than that of the main population of the CD8⁺bright T lymphocytes (24).
As shown in Figure 5A, in FCM, all the cells with CD8-ECD intensity above that of the large cluster of negative stained cells are counted as CD8⁺ T lymphocytes. The CD8⁺dim T lymphocytes are those cells that fall between the large cluster of negative and the large cluster of bright positive cells.

To investigate whether the SP ICM can detect fluorescence signals as low as that from the CD8⁺dim cells, four groups of PerCP-magnetic beads with different intensities were prepared by biotin-streptavidin conjugation and their fluorescent GIs were quantified by the FCM and by our SP ICM. We named the groups PerCP-1 to PerCP-4, where the lowest intensity group is PerCP-1 and the highest intensity one is PerCP-4.

Table 1. Comparison of Log₁₀(GI) (GeoMean intensity) of PerCP fluorescence of PerCP-magnetic beads with CD8⁺bright T lymphocytes and CD8⁺dim T lymphocytes obtained by FCM and by SP ICM (5 sec exposure time).

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<tr>
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<th>FCM</th>
<th>SP ICM</th>
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<tr>
<td>PerCP-1</td>
<td>1.44</td>
<td>4.28</td>
</tr>
<tr>
<td>PerCP-2</td>
<td>2.18</td>
<td>4.69</td>
</tr>
<tr>
<td>PerCP-3</td>
<td>2.36</td>
<td>4.89</td>
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<td>PerCP-4</td>
<td>2.94</td>
<td>5.46</td>
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<tr>
<td>CD8⁺bright T cells</td>
<td>2.60 (2.30 – 2.85)</td>
<td>5.22</td>
</tr>
<tr>
<td>CD8⁺dim T cells</td>
<td>1.64 (1.18 – 2.30)</td>
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Table 1 shows the comparison of Log₁₀(GI) of the PerCP fluorescence of the PerCP-magnetic beads with those of CD8⁺bright T lymphocytes and CD8⁺dim T lymphocytes. Log₁₀(GI) values obtained by FCM and by SP ICM (5 sec exposure time) were given in the table. The FCM data indicate that the Log₁₀(GI) of CD8⁺bright T lymphocytes is between that of the group PerCP-3 and that of the group PerCP-4, and the Log₁₀(GI) of CD8⁺dim T lymphocytes is close to that of the group PerCP-1. In the SP ICM, the Log₁₀(GI) of CD8⁺ T lymphocytes (all detected CD8⁺ T lymphocytes) is close to that of the group PerCP-4.

To investigate whether the SP ICM could simultaneously detect CD8⁺bright T lymphocytes and CD8⁺dim T lymphocytes, a known number of the beads of groups PerCP-1 and PerCP-4 were mixed and added to a whole blood sample, to mimic the
CD8PerCP labeled CD8\(^{\text{dim}}\) T lymphocytes and CD8\(^{\text{bright}}\) T lymphocytes. As indicated in the Materials and Methods section, the sample was prepared as usual for the SP ICM. In the image obtained by the SP ICM, only the group PerCP-4 is present. For a sample with a mixture of the group PerCP-2 and PerCP-4, beads from both two groups are shown in the image. These results demonstrate that the SP ICM can not detect the complete range of CD8\(^{\text{dim}}\) T lymphocytes when also CD8\(^{\text{bright}}\) T lymphocytes are present. Apparently only the CD8\(^{\text{dim}}\) T lymphocytes with a relatively high intensity are counted.

**Comparison of SP ICM with SP FCM**

![Graphs showing comparison of SP ICM with SP FCM](#)
FIG. 6. Linear regression plot (A) and Bland-Altman plot (B) of CD4+ T (A1, B1), CD8+ T (A2, B2) lymphocytes enumerations and CD4/CD8 ratio (A3, B3) using the SP FCM (tetraCXP) and the SP ICM. Blood specimens of 45 HIV− children were tested. The blood specimens of 50 HIV− adults, 50 HIV+ adults and 45 HIV− children were tested using the SP ICM method and the SP FCM (tetraCXP method), and the obtained CD4+ T and CD8+ T lymphocytes counts and CD4/CD8 ratios were compared with each other. In the SP ICM, the trained operator manual threshold method was used for image analysis. Figure 6 shows the linear regression plot (A) and the Bland-Altman plot (B) of CD4+ T (A1, B1), CD8+ T (A2, B2) lymphocytes enumeration and CD4/CD8 ratio (A3, B3) of blood specimens of 45 HIV− children. (There are 2 children with CD4/CD8 ratio lower than 1: one is 5 year old and the other is 6 years old. This ratio is not an indicator of HIV infection. As shown in Figure 1B, the CD4/CD8 ratio of 1 is used as an indicator of HIV infection only for children younger than 2 years old.) In the FCM method, the standard automatic CD8 gating was applied, as shown in Figure 5A. Similar graphs were obtained on blood specimens of 50 HIV− and 50 HIV+ adults. From these graphs the slope and correlation coefficient ($R$) from linear regression plot (X axis: SP FCM; Y axis: SP ICM) and the bias, 95% CI of bias, and limits of agreement from Bland-Altman plot (%) in each comparison group were obtained and listed in Table 2.

The results given in Table 2 indicate that for CD4, CD8 count and CD4/CD8 ratio, good correlations were obtained between the SP ICM and the SP FCM for blood specimens of 50 HIV− adults, 50 HIV+ adults and 45 HIV− children ($R$: 0.94 − 0.99). For CD4 enumeration of 50 HIV− adults and 45 HIV− children, the results indicated that the SP ICM was comparable to the SP FCM with a bias of −10%. For CD4 enumeration of 50 HIV+ adults, the slope (0.99) and the bias (1.0%) indicated that the SP ICM was comparable to the SP FCM. For CD8 enumeration of 50 HIV− adults and 45 HIV− children, the results indicated that the SP ICM was comparable to the SP FCM with a bias of −10%. For CD8 enumeration of 50 HIV+ adults, a slope of 0.81 and a bias of -21.3% were obtained.
Table 2. Comparison of the SP ICM (trained operator manual threshold method) with the SP FCM (tetraCXP). In the SP FCM method, the standard automatic CD8 gating was applied. a Slope and correlation coefficient ($R$) from the linear regression plots; b Bias, 95% confidence interval (CI) of bias, and limits of agreement from the Bland-Altman plots (%).

<table>
<thead>
<tr>
<th>Parameters</th>
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<th>$R$ a</th>
<th>Bias b (%)</th>
<th>95% CI of bias b (%)</th>
<th>Limits of agreement b (%)</th>
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<td>HIV− children (45)</td>
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For the three patient groups tested, the CD8 results tend to be somewhat lower than in FCM, whereas the CD4 tends to be somewhat higher. Consequently, results of the SP ICM for the CD4/CD8 ratios of 50 HIV− adults, 45 HIV− children and 50 HIV+ adults are about 20% higher than in SP FCM.

Counting only CD8 bright T lymphocytes in FCM

We investigated whether the lower CD8 count in the SP ICM was partly due to CD8 dim T lymphocytes that are not counted in the SP ICM because of its limited sensitivity. The FCM data were re-analyzed and at this time only CD8 bright T lymphocytes were counted. Next the CD8 bright count was compared to the CD8 count obtained by the SP ICM method.

Figure 5A illustrates the automatic gating in the FCM for CD8+ T lymphocytes counting (Scatter plot: CD8-ECD vs. CD3-PC5), in which all the cells with CD8-ECD intensity above 1 are counted as CD8+ T lymphocytes. Figure 5B illustrates the manual gating used for the CD8 bright T lymphocytes count (Histogram: counts vs. CD8-ECD).
CD3-PC5 positive cells are plotted in the histogram (counts vs. CD8-ECD). The CD8\textsuperscript{bright} T lymphocytes are gated in the symmetric region of the CD8\textsuperscript{+} histogram peak. The rest of the cells above intensity 1 are gated as CD8\textsuperscript{dim} T lymphocytes. In our study, it was found that on average, CD8\textsuperscript{+} T lymphocytes were about 10% of total CD8\textsuperscript{+} T lymphocytes for all groups, i.e. HIV\textsuperscript{−} adults, HIV\textsuperscript{+} adults and HIV\textsuperscript{−} children.

Then only CD8\textsuperscript{bright} T lymphocytes were counted in the FCM and the obtained CD8\textsuperscript{bright} counts and CD4/CD8\textsuperscript{bright} ratios were compared with the CD4/CD8 ratios obtained by the SP ICM. Table 3 shows the slope and correlation coefficient (R) from the linear regression plot (X axis: SP FCM; Y axis: SP ICM) and the bias, 95% CI of the bias, and limits of agreement from the Bland-Altman plot (%), for each comparison group. The blood specimens of 30 of the 50 HIV\textsuperscript{−} adults, of 38 of the 50 HIV\textsuperscript{+} adults, and of 28 of the 45 HIV\textsuperscript{−} children were re-analyzed. The results of Table 3 shows that now for the CD4, CD8 counts and CD4/CD8 ratios, good correlations were obtained between the SP ICM data and the SP FCM data. These results indicate that the main reason of the somewhat lower CD8 count is most likely the ~ 10% CD8\textsuperscript{dim} T lymphocytes that are not counted in the SP ICM.

**Table 3.** Comparison of the SP ICM with the SP FCM (tetraCXP). In the SP FCM method, only CD8\textsuperscript{bright} T lymphocytes were gated. \(^a^\) Slope and correlation coefficient (R) from the linear regression plots; \(^b^\) Bias, 95% confidence interval (CI) of bias, and limits of agreement from the Bland-Altman plots (%).

<table>
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<th>95% CI of bias (^b^) (%)</th>
<th>Limits of agreement (^b^) (%)</th>
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Comparison of Different Image Analysis Algorithms in SP ICM

FIG. 7. Linear regression plot (A) and Bland-Altman plot (B) of CD4+ T lymphocytes enumerations using the SP FCM (tetraCXP) and the SP ICM. In the SP ICM, CD4PE images were analyzed by the trained operator manual threshold method (A1, B1), the dynamic threshold method (A2, B2) and the maximum entropy threshold method (A3, B3), respectively. Blood specimens of 50 HIV+ adults were tested.
To test the capability of two new image analysis algorithms, the dynamic threshold method and the maximum entropy threshold method, the CD4PE images from the blood specimens of 50 HIV− adults, 50 HIV+ adults and 45 HIV− children obtained by the SP ICM were also analyzed using these two methods. The results by these two new algorithms and by the trained operator manual threshold method were compared with those obtained by the SP FCM (tetraCXP). Figure 7 shows the linear regression plot (A) and Bland-Altman plot (B) of CD4+ T lymphocytes enumeration of blood specimens of 50 HIV+ adults using the SP FCM (tetraCXP) and the SP ICM. In the SP ICM, CD4PE images were analyzed by the trained operator manual threshold method (A1, B1), the dynamic threshold method (A2, B2) and the maximum entropy threshold method (A3, B3), respectively. Similar plots were obtained for blood specimens of 50 HIV− adults and 45 HIV− children. All data are summarized in Table 4. The three methods gave comparable results. Among them, the dynamic threshold method gave a slightly lower $R$ and a slightly larger range of limits of agreement. The entropy threshold method gave almost identical results as those by the trained operator manual method. As mentioned in Materials and Methods, this method is also the fastest one tested. Based on these results this method will be implemented in the instrument.
Table 4. Comparison of the CD4 counts obtained respectively by the trained operator manual threshold method, the dynamic threshold method and the maximum entropy threshold with those obtained by the SP FCM (tetraCXP). a Slope and correlation coefficient ($R$) from the linear regression plots; b Bias, 95% confidence interval (CI) of bias, and limits of agreement from the Bland-Altman plots (%).

<table>
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</table>

**DISCUSSION**

The SP ICM was tested by determinations of CD4+ T and CD8+ T lymphocytes counts and CD4/CD8 ratios. The system was evaluated by comparison with the SP FCM tetraCXP method on blood specimens of 50 HIV− adults, 50 HIV+ adults and 45 HIV− children.

Most importantly, for all three patient groups the CD4 values measured agreed very well with the SP FCM data. Both the results of the correlation analysis and the Bland-Altman plots showed that the SP ICM data correlate well with the FCM data for CD4. This also holds in those low concentration ranges that are very important for diagnosis and treatment, i.e. below 350, and especially below 200 CD4+ T lymphocytes per μl.
In counting CD8+ T Lymphocytes a distinction has to be made between the CD8$^{\text{bright}}$ cells and the CD8$^{\text{dim}}$ cells. It was shown that the sensitivity of the current SP ICM method is such that the CD8$^{\text{dim}}$ cells are not, or partly not, counted. The reasons may be the relatively high background of free fluorophore present in the system, or the inability of the counting algorithm to distinguish between CD8$^{\text{dim}}$ cells and the cross-talk of CD4PE fluorescence. It was shown that the CD8 counts are therefore about 10% lower than those by the SP FCM. This may at least partly explain why the CD4/CD8 ratio is about 20% higher than found in the FCM. One has to be careful however about these data. The systematic errors in comparing absolute count data for different FCM instruments may easily reach 20% or be even higher (25, 26). This certainly holds where completely different methods and instruments are compared. The CD8 counts and CD4/CD8 ratios we found did show good linear relations with the FCM data, and a good interchangeability between the two methods indicated by the Bland-Altman plots.

It should be noted that in literature similar differences in enumerating CD8 cells have been reported, as e.g. for the Guava PCA method (27), which has a limited sensitivity and gate settings that only counts CD8$^{\text{bright}}$ T lymphocytes.

Several options for further improvement may be implemented in the future. An obvious way to prevent the interference of the cross-talked CD4PE cells on CD8$^{\text{dim}}$ T lymphocytes enumeration is to perform CD4 and CD8 enumeration in two separate analysis chambers. However, this will increase the cost per test and double the sample preparation steps. Another way may be to calculate a correction starting from the (measured) PE images. The limited sensitivity of the SP ICM system in the detection of low intensity fluorescence is due to the presence of free fluorophore and ferrofluid in the sample. The resulting high background compresses the dynamic range of the system to a much higher degree than in FCM where no ferrofluid is present and the free fluorophores are diluted. In the current SP ICM system, a sample chamber with inner depth of 4mm was used. A preliminary test, using a thin chamber with ~140 μm depth and the PerCP beads with different intensities, was performed; the results indicated that the whole range of CD8$^{\text{dim}}$ T lymphocytes can be detected, when CD8$^{\text{bright}}$ T lymphocytes are also present.
It was found from this study that the slopes comparing the SP ICM to the SP FCM of both CD4 and CD8 counts of HIV$^-$ adults and HIV$^-$ children are ~ 10% higher than those of the HIV$^+$ adults, as shown in Table 2. This phenomenon could be related to the fact that the expression of CD3, CD4, CD8 receptors on T lymphocytes is significantly decreased for HIV infected patients (28). It may be that some T lymphocytes with less CD3 receptors were not magnetically attracted to the surface within 20 min magnetic separation. Adding more CD3FF and longer magnetic separation time may help.

In order to have a real automatic system for less trained operators, two new image analysis algorithms, the dynamic threshold method and the maximum entropy method were developed and tested. They both performed well, and were comparable with the FCM data and the results obtained by the trained operator manual threshold method. The dynamic threshold method showed a slightly larger deviation and it took much longer analysis time. The maximum entropy threshold method is more accurate and works faster. This method will be implemented in the instrument.

In conclusion, our system is a good candidate for pediatric HIV monitoring in point-of-care settings of resource-constrained countries. It is clear however that a larger test, involving more patients and also pediatric HIV$^+$ children has to be performed. The field test was performed in Thailand and the results were given in chapter 7 of this thesis.

ACKNOWLEDGEMENTS

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REFERENCES

Chapter 5


CHAPTER 6

A Novel Affordable CD4 Enumeration Method: Discriminating Phagocytes by Activation

ABSTRACT

Background: HIV monitoring in resource-constrained settings demands affordable and reliable CD4+ T lymphocytes enumeration methods. We developed a novel, affordable CD4 enumeration method by combining immuno-magnetic selection of CD4 positive cells using only CD4 antibody and a cell activation strategy to discriminate the co-selected monocytes and granulocytes. A dedicated simple single platform image cytometer (SP ICM) was built, which is designed to be low-cost, yet reliable and robust. In this paper we present the immunocytochemical methodology, the SP ICM and test the method on blood samples from healthy donors and HIV positive patients.

Methods: Whole blood samples were treated with a DNA dye Hoechst33342, the oxidative burst stimulator Phorbol 12-myristate 13-acetate, and Dihydrorhodamine123. By CD4 immuno-magnetic labeling, CD4+ T lymphocytes, CD4+dim monocytes and some non-specifically bound granulocytes are magnetically attracted to an analysis surface of a chamber with known volume. Two fluorescent images of the same area are recorded and analyzed: a Hoechst33342 image (DNA image) shows all magnetically attracted leukocytes at the surface, and a Rhodamine123 image shows activated monocytes and activated granulocytes (mainly neutrophils). By subtracting the cell counts of these 2 images, the absolute CD4+ T lymphocytes count can be obtained.

Results: The principle of our CD4 enumeration system was verified on blood samples of 29 healthy donors using a fluorescence microscopy. No difference in the oxidative burst

activation kinetics between 42 HIV+ patients and 18 healthy donors was found using flow cytometry. Furthermore, the dedicated SP ICM instrument was built and evaluated by comparing the cell counts of blood samples of 42 HIV+ patients with the counts obtained by SP FCM. A reasonably good correlation was achieved. Bland-Altman plots showed interchange-ability between our SP ICM method and the SP FCM methods.

Conclusions: This CD4 enumeration method is affordable, simple and easy-to-handle even for less trained operators. It is a good candidate for CD4+ T lymphocytes enumeration in point-of-care settings in resource-constrained countries.

INTRODUCTION

The speedy increase of the incidence of Human Immunodeficiency Virus (HIV) infection in Africa and other countries demands a wide application of CD4 enumeration technologies to monitor the HIV infection stage in patients. For point-of-care settings in resource-constrained countries, flow cytometry (FCM), the gold standard for CD4 enumeration, is too expensive and requires well trained personnel that is often not available. CD4 enumeration systems, being affordable, simple, easy-to-handle, solid, and reliable are therefore urgently required.

Previously, an immuno-magnetic single platform image cytometer (SP ICM) based on antibody specificity was built in our group. This system, named StarCount 1.0, is used for enumeration of cell types that are well defined by a single type of cell surface antigen. Excellent correlation with FCM was achieved for white blood cells (CD45), T lymphocytes (CD3) and B lymphocytes (CD19). However, for CD4 enumeration, this system was not applicable, especially in case of Acquired Immunodeficiency Syndrome (AIDS). This arises because the CD4 receptor is also present on monocytes (1). Later on we developed a dedicated CD4 enumeration SP ICM, StarCount 2.0. This system applies 2 monoclonal antibodies, CD3 and CD4: One for immuno-magnetic selection and the other for immuno-fluorescent labeling (2, 3). CD3+CD4+ T lymphocytes are enumerated, whereas monocytes and nonspecifically adhered granulocytes are excluded. It was proven that this system can reliably determine the CD4 count. However, an obvious
drawback of this system for application in resource-poor settings is the use of two (expensive) monoclonal antibodies.

Oxidative burst is a characteristic response of phagocytes. In response to a variety of stimuli e.g. pathogens, human phagocytes (neutrophils that form about 95% of granulocytes, monocytes and macrophages) activate the NADPH oxidase system and produce reactive oxygen species (ROS) in an oxidative burst process (NADPH + 2O₂ → NADP⁺ + 2O₂⁻). The ROS plays a critical role in killing bacteria and fungi. Upon treatment with Phorbol 12-myristate 13-acetate (PMA), which is a potent stimulus of the oxidative burst, NADPH oxidase is activated and ROS is generated (4 - 7). The ROS can be determined using Dihydrorhodamine123 (DHR123), a non-fluorescent, cell membrane-permeable compound that localizes in the mitochondria. Intracellular DHR123 is oxidized by the ROS in activated phagocytes, and is converted to green fluorescent Rhodamine123 (R123), which is membrane-impermeable and is sequestered by active mitochondria (8, 9).

Applying immuno-magnetic CD4 labeling and PMA cell activation, we designed a novel, affordable CD4 enumeration system. This system applies only CD4 monoclonal antibodies to immuno-magnetically label all CD4⁺ cells which are then magnetically collected at an analysis surface of a chamber with known volume. This surface contains CD4⁺ T lymphocytes, CD4⁺⁺ monocytes and nonspecifically labeled granulocytes. By recording a DNA fluorescence image (Hoechst33342) all these cells at the surface are counted. Because of PMA stimulation, a second fluorescent image of R123 containing cells can be recorded. It shows the activated monocytes and granulocytes, which can be counted from this image. From the difference of the cell counts of these two images we derive an absolute CD4 count.

First the immunocytochemical methodology was tested using fluorescence microscopy and flow cytometry. The principle of our system was verified on fresh blood samples of 29 healthy donors. A quantitative fluorescence microscopy equipped with a CCD camera was used for image recording and a Labview program for image analysis. The obtained CD4 counts were compared with those from the single platform flow cytometer (SP FCM) TruCount method. A good correlation coefficient and a reasonable slope were
obtained. Furthermore, the oxidative burst activation kinetics of 42 HIV+ patients and 18 healthy donors were compared using flow cytometry. No difference was found, indicating that our method can be used for testing on HIV+ patients.

Next a dedicated CD4 enumeration instrument, StarCount 3.0 (SC 3.0) suited for the immunocytochemical collection and counting procedure outlined above was built and evaluated using fresh blood specimens of 42 HIV+ patients. CD4 counts obtained by our system and by SP FCM tetraCXP method show a good agreement.

MATERIALS AND METHODS

Blood Collection

Blood samples from randomly selected adult HIV+ patients were supplied by MST hospital, Enschede, The Netherlands. Blood samples from healthy donors were obtained from adult volunteers. Unless mentioned elsewhere, blood samples were collected in sterile K$_3$EDTA blood collection tubes (BD Biosciences, USA) by venipuncture and processed within 8 hrs after draw.

Oxidative Burst Stimulation and Activation Kinetics

To 100 μl EDTA-anticoagulated whole blood, 2 μl of PMA (1 mM; Sigma-Aldrich, USA) and 12.5 μl of Dihydrorhodamine123 (DHR123, 1mM; Molecular Probes, USA) were added. The mixture was incubated in the dark, at RT or at 37 °C. At predetermined time points of reagents incubation, i.e. 5, 15, 25, 35 and 45 min, 10 μl sample was taken and diluted with 340 μl system buffer and analyzed on a FACSCalibur using CELLQUEST™ software (BD Bioscience, USA).

To determine the kinetics of the activation, the Rhodamine123 (R123) intensities of neutrophils and monocytes were measured by Cytomics FC500 using tetraCXP software (Beckman Coulter, USA) at 25 min, 35 min, 45 min and 55 min of reagents incubation time.

Approximately 5000 leukocytes were collected from each blood sample. Neutrophils and monocytes were gated using forward-scatter vs. side-scatter dot plots, and using
R123 intensity vs. side-scatter dot plots. Mean R123 intensity and standard deviation of both the neutrophil and the monocyte populations were recorded.

For neutrophil and monocyte count, either TruCount™ absolute count tubes (BD Bioscience, USA) were used on a FACSCalibur or Flow-Count Fluorospheres (Beckman Coulter, USA) were used on a Cytomics FC500 (10, 11).

**CD4 Enumeration and Immunophenotyping by Fluorescence Microscopy**

100 µl of EDTA-anticoagulated whole blood was mixed with activation agents (2 µl of 1mM PMA and 12.5 µl of 1mM DHR123), 4 µl of 1mg/ml Hoechst33342 (Molecular Probes, Invitrogen, USA) and 15 µl of EasySep human CD4 cocktail (clone: QS4120, isotype: Mouse IgG1, StemCell Technologies, Canada). After 15 min incubation in the dark at RT, 7.5 µl of EasySep magnetic nanoparticles were added and incubated for 10 min in the dark at RT. For CD4 enumeration, the sample was diluted with system buffer to a final volume of 410 µl. About 350 µl of sample solution was transferred into a sample chamber and placed in a MagNest® (Immunicon Inc., USA). After 20 min magnetic incubation, the sample chamber/magnet assembly was put on the stage of a fluorescence microscopy (Nikon ECLIPSE E400, Japan). Fluorescent Hoechst33342 and R123 images were respectively recorded from 5 different image areas (0.532 µl whole blood/image). A Labview program was applied to count the cells in the images. The CD4 count was obtained by subtracting the cell count of the R123 image from the cell count of the Hoechst33342 image.

In case of immunophenotyping, the blood specimens were treated in the same manner except that 20 µl of CD14APC-Cy7 (BD Bioscience, USA) was added to blood samples before diluting to 410 µl. Fluorescent Hoechst33342, R123, and APC-Cy7 images were recorded, showing respectively all the leukocytes at the surface, the activated monocytes and granulocytes, and only the monocytes.

**CD4 Enumeration by FCM**

For CD4 enumeration using the TruCount method (FACSCalibur, BD Bioscience, USA), TruCount™ absolute count tubes were used and blood samples were processed
using a lyse-no-wash method according to the manufacturer’s recommendation (10). Reagents used were CD3 (clone: SK7, isotype: mouse IgG1-κ)-FITC/CD4 (clone: SK3, isotype: mouse IgG1-κ)-PE/CD45 (clone: 2D1, isotype: mouse IgG1-κ)-PerCP (TriTEST, BD Bioscience, USA). The samples were analyzed on a FACSCalibur using CELLQUEST™ software.

For CD4 enumeration using the TetraCXP method (FC500, Beckman Coulter, USA), samples were prepared according to the manufacturer’s recommendation (11). To 100 μl of EDTA anti-coagulated whole blood, 5 μl of Cyto-stat tetraCHROME (CD45 (clone: B3821F4A, isotype: mouse IgG2b)-FITC/CD4 (clone: SFCI12T4D11, isotype: mouse IgG1)-RD1/CD8 (clone: SFCI21Thy2D3, isotype: mouse IgG1)-ECD/CD3 (clone: UCHT1, isotype: mouse IgG1)-PC5) was added and mixed. (FITC, fluorescein isothiocyanate; RD1, R-phycoerythrin; ECD, R-phycoerythrin/Texas Red tandem dye; PC5, R-phycoerythrin/cyanine5 tandem dye.) After 10 to 12 min incubation in dark at RT, the sample was lysed and fixed in a Multi-Q-Prep workstation. 100 μl of Flow-Count Fluorospheres was added and mixed before analysis. The samples were analyzed on a Cytomics FC500 using tetraCXP software, and absolute CD4 counts were obtained. These tests were performed in the MST hospital, Enschede, The Netherlands.

**CD4 Enumeration by StarCount 3.0**

Samples were prepared as used for fluorescence microscopy but CD4-ferrofluid (CD4FF, 0.88 mg/ml, clone: EDU-2, isotype: mouse IgG2a-κ, Immunicon Inc., USA) instead of EasySep CD4, was used for immunomagnetic labeling. Only 1 step of reagents incubation is needed for CD4FF, which makes sample preparation simple. To 100 μl EDTA anti-coagulated human whole blood, 2 μl of CD4FF, 8 μl of 1 mg/ml Hoechst33342, 2 μl of 1 mM PMA, and 12 μl of 1mM DHR123 were added and mixed. After 15 min incubation in the dark at RT, the sample was diluted with system buffer to a final volume of 400 μl and mixed. Approximately 350 μl of sample solution was transferred into the chamber and the chamber was inserted into a MagNest®. After 20 min magnetic incubation, the sample chamber was inserted into the StarCount 3.0. Fluorescent images of Hoechst33342 and R123 were recorded.
Hoechst33342 is a cell membrane-permeable vital DNA dye. It stained all leukocytes brightly and homogeneously. Hoechst33342 is excited maximally by UV light at 350 nm, and the maximum of the emission is in the blue at 461 nm (Molecular Probes, Invitrogen, USA); this has no interference with R123 emission (maximum excitation at 505 nm; maximum emission at 533 nm). The exposure time was 1 second for R123, and 10 seconds for Hoechst33342. For each test, six images from 3 separate positions on the analysis surface were recorded. Each image corresponds to a volume of 1.096 μl of whole blood. The total volume (3.288 μl) was chosen to obtain a theoretical statistical Poisson variation of approximately 5.5% at 100 cells/μl and approximately 7.8% at 50 cells/μl.

Accuracy and Linearity of StarCount 3.0 System

To evaluate the accuracy and linearity of our system within the clinically important range of CD4⁺ T lymphocytes counts (0 ~ 600/μl) (12 - 15), a series of diluted blood samples containing a known number of CD4⁺ T lymphocytes were prepared according to the following protocol: First a CD4 enumeration was performed using our system on a whole blood specimen of a healthy donor. Then the original whole blood specimen was diluted with system buffer in different ratios, resulting in a series of 6 specimens with known numbers of CD4⁺ T lymphocytes (21, 31, 72, 144, 310 and 609/μl, measured by the TruCount method). The CD4⁺ T lymphocytes count of the whole blood specimen and that of each diluted blood samples were determined by our system for 5 replicates. The accuracy and the linearity were evaluated. As a reference, CD4⁺ T lymphocytes counts of the above mentioned samples were also measured by the TruCount method.

Statistical Analysis

Based on the CD4⁺ T lymphocytes counts obtained from different methods, linear regression lines were drawn and correlation coefficients (R) were calculated. Bland-Altman plots (16 - 18) were used to evaluate the interchange-ability between methods. In Bland-Altman plots, the average of the CD4 counts obtained from two methods is plotted on the horizontal axis, and the difference/average% is plotted on the vertical axis. The solid line in the plot represents the bias (the average difference between
the two methods), and the dashed lines in the plot illustrate the upper and lower limits of agreement (± 1.96 SD).

RESULTS

Cell Activation

The cell activation strategy for our novel CD4 enumeration method was first tested methodologically for healthy donors. The oxidative burst of neutrophils and monocytes was stimulated by PMA and stained with Hoechst33342 and R123, as described in materials and methods. Figure 1 shows a fluorescence microscopy image of 2 lymphocytes and 1 neutrophil after PMA stimulation for 35 min. The neutrophil, which can be identified by its multi-lobed nucleus, shows very bright R123 fluorescence. On the contrary, lymphocytes, which can be identified by their spherical nuclei, are not stained with R123. Monocytes (not shown here) can clearly be recognized by the kidney shape of the nucleus and the R123 fluorescence, which is not as bright as that of the neutrophils.

The R123 fluorescence intensity of neutrophils and monocytes was quantitatively studied using FCM. Figure 2 gives an example of the FCM dot plots of R123 intensity vs. side-scatter (SSC) of a blood sample without PMA stimulation (A), and of a sample stimulated with PMA (B). In both cases, the R123 intensity was measured after 35 min of reagents incubation. Without PMA stimulation, the R123 intensity of the neutrophils is only slightly higher than that of the lymphocytes and the background (red blood cells). The R123 intensity of the monocytes is higher than that of the neutrophils and the lymphocytes. After stimulation with PMA, the R123 intensity of neutrophils and monocytes has increased dramatically. Now the neutrophils show a higher R123 intensity than the monocytes. In comparison, lymphocytes are still R123 negative. These results agree well with the staining in the fluorescent image shown in Figure 1.

Figure 3 shows the mean R123 intensity of different cell types (neutrophils, monocytes and lymphocytes, respectively) detected by FCM as a function of incubation time with PMA. The control curves represent the data without Hoechst33342 staining (only PMA and DHR123 were applied for incubation) and the curves represent the case with Hoechst33342 staining were also shown. In both cases, neutrophils and monocytes
showed an increase of the R123 intensity with time; while lymphocytes did not show detectable R123 intensity. Furthermore, at each time point and for each cell population, the measured R123 intensity of cells stained by Hoechst33342 was the same as that without Hoechst33342 staining. These results show that Hoechst33342 has no effect on the oxidative burst.

**FIG. 1.** A fluorescence microscopy image of 2 lymphocytes and 1 neutrophil after PMA stimulation for 35 min. The DNA was stained with Hoechst33342 (blue) and activated cells show R123 (green) fluorescence. (color figure on Page 213)

**FIG. 2.** FCM dot plots (R123 intensity vs. side-scatter (SSC)) of a blood sample without PMA stimulation (A), and of a sample stimulated with PMA (B).
We conclude from the tests above that the oxidative burst can be applied to distinguish monocytes and granulocytes (mainly neutrophils) from CD4$^+$ T lymphocytes.

**CD4 Enumeration for Healthy Donors**

The principle of the CD4 enumeration system applying phagocytes activation was evaluated on blood samples of 29 healthy donors. EasySep Human CD4 Selection reagent was applied to magnetically select CD4$^+$ cells. Blood samples were also incubated with Hoechst33342 and DHR123. Both a Hoechst33342 image and a R123 image were recorded.

In the Hoechst33342 image, all the magnetically selected leukocytes were stained homogeneously and could be counted accurately; in the R123 image, bright cells (granulocytes, mainly neutrophils) and dim cells could be distinguished. By using different values of parameter settings in our Labview program, all R123 cells and only bright R123 cells were counted respectively.

After stimulation of an oxidative burst, monocytes show a mediate R123 intensity (FCM). In order to determine the cell type of the dim R123 cells, we characterized the...
magnetically selected monocytes by immuno-phenotyping using CD14APC-Cy7. The CD14APC-Cy7 image was overlain on the R123 image and the monocytes (CD14APC-Cy7+ cells) were compared with the R123 dim cells. The results showed that 98.8% of the monocytes were R123 dim (322 out of 326), only two (0.61%) of the monocytes were R123 bright and two (0.61%) were R123 negative. On the other hand, 94.4% of the R123 dim cells were monocytes. The rest 5.6% could be less active neutrophils. The results show that not only non-specifically bound granulocytes, but also almost all of the magnetically selected CD4dim monocytes can be distinguished from CD4+ T lymphocytes.

The absolute CD4 count was calculated by subtracting the R123 cell count from the Hoechst33342 cell count. The absolute CD4 counts were then compared with those obtained by SP FCM TruCount. The linear regression and Bland-Altman plot of the CD4 counts obtained by these two methods are shown in Figure 4. A good correlation was obtained ($R = 0.93$, slope = 0.95, see Figure 4a). The Bland-Altman plot shows interchange-ability between the two methods, with a bias of $-4.5\%$ and limits of agreement from $-22.7$ to $13.7\%$ (Figure 4b).

The percentage of R123 bright neutrophils and that of R123 dim monocytes in the total number of magnetically selected cells were calculated respectively for each healthy donor. The percentage of the non-specifically bound neutrophils ranges from 1% to 38%, and that of CD4dim monocytes ranges from 2% to 15%. The percentage of R123 bright granulocytes (mainly neutrophils) from each donor was plotted against the corresponding count as obtained by FCM (FACSCalibur) in the whole blood count. Figure 5 shows that the percentage of neutrophils in the magnetically selected cells was independent of its cell count in the whole blood. For monocytes, similar results were obtained (data not shown).

We conclude that the principle of the alternative CD4 enumeration method has been verified.
FIG. 4. Linear Regression (A) and Bland-Altman plot (B) of CD4 count obtained by fluorescence microscopy (SP ICM) and by SP FCM TruCount method for blood samples of 29 healthy donors.

FIG. 5. Percentage of R123 bright granulocytes (mainly neutrophils) from each donor was plotted against the corresponding granulocytes (mainly neutrophils) count in the whole blood sample as obtained by FCM (29 healthy donors).

Kinetics of Oxidative Burst Activation for Healthy Donors and HIV+ Patients

To investigate whether the activation method to enumerate CD4+ T lymphocytes works equally well for both healthy donors and HIV+ patients, we compared the kinetics of the oxidative burst for 18 healthy donors and 42 HIV+ patients. For the patients the CD4 counts ranged from 59 to 853/µl (FCM). Figure 6 shows the mean and the standard deviation of the measured R123 intensities as a function of reagents incubation time. No
difference was found in the activation kinetics for these two groups. This indicates that the activation principle may be applied for CD4 enumeration of HIV+ patients.

FIG. 6. Comparison of oxidative burst activation kinetics between blood samples of 18 healthy donors and of 42 HIV positive patients using FCM (FC500). The mean R123 intensity and the standard deviation of intensities at each reagents incubation time point are shown.

Instrumentation

FIG. 7. A schematic drawing of the main components in our CD4 enumeration instrument.
We built a prototype of an instrument for CD4 enumeration by using the cell activation strategy, StarCount 3.0. The design was in part the same as that of the previously developed StarCount 2.0 instrument (2, 3). Figure 7 illustrates schematically the main components of StarCount 3.0. CD4 immuno-magnetically labeled cells are subjected to a homogenous magnetic force pointing into the positive Z-direction of the magnetic chamber (1, 19, 20). This drives the cells to the upper glass surface of the chamber (analysis surface) where they are deposited. The fluorescence of the CD4 immuno-magnetically attracted cells is excited by two pairs of Light Emitting Diodes (LEDs). Each pair of LEDs is symmetrically mounted above the magnet. Two LEDs (6 mW, 375 nm, NS375L-5RLO, Roithner LaserTechnik, Austria) are used for excitation of Hoechst33342, no excitation filter is applied. The other two LEDs (3 W, 493 nm, Lumileds Luxeon III Star Lambertian Blue, Future Electronics, UK) are used for excitation of R123. In front of each LED we placed an excitation filter (470AF50, Omega optical, USA) to suppress the light emitted by the LED in the region overlapping with the emission of R123. The emission is collected by a 10 × objective (NA 0.2, Lomo Optics, USA), and filtered by an appropriate emission filter mounted in a filter changer. Two emission filters are mounted: one for Hoechst33342 (420LP, GG420, Schott AG, Germany), and the other for R123 (535AF45, Omega optical, USA). Then the filtered fluorescent signal is focused onto an ST-402ME CCD camera (SBIG, USA). The 2 pairs of LEDs can alternatively be switched on manually; the emission filters can be changed accordingly. The images recorded are transferred to a single board computer (EES-3610, EvalueTech, Taiwan) with a touch-screen monitor (B084SN03 V2, AU Optronics, Taiwan), and analyzed using a dedicated freeware image analysis algorithm that determines the number of cells per μl of whole blood. The dimensions of our StarCount 3.0 instrument are 25 cm × 25 cm × 20 cm. The system operates on 100 ~ 250 V, 47 ~ 63 Hz, or on one 12 V rechargeable battery.

Absolute CD4 counts can be obtained by subtracting the number of cells in the R123 image from the number of cells in the Hoechst123 image. Figure 8 shows a typical 2-color merge image of one area in the analysis surface. There is a shift of a few pixels between these 2 images. As expected, each R123 spot has a corresponding dot in the
Hoechst33342 image. Also, bright R123 cells (neutrophils) and dim R123 cells (monocytes) can be distinguished.

FIG. 8. A typical 2-color merge image of one area in the analysis surface. Red represents Hoechst33342 staining, and green represents R123 staining. There is a shift of a few pixels between these 2 images. A: Two cells with dim R123 staining and corresponding Hoechst33342 staining; B: Two cells with bright R123 staining and corresponding Hoechst33342 staining; C: Two cells with Hoechst33342 staining but without R123 staining; D: One cell with bright R123 staining and another cell with dim R123 staining. (color figure on Page 213)

Accuracy and Linearity of SC 3.0

To evaluate the accuracy and linearity of the system, a whole blood sample from a healthy donor and a series of diluted samples containing graded, known numbers of CD4+ T lymphocytes were prepared. All samples were checked in parallel by the "gold standard" BD TruCount method (10) and the SC 3.0. Figure 9 shows the CD4 count obtained by our system versus the CD4 count obtained by the TruCount method. A linear relation ($R = 0.99; \text{slope} = 1.17$) was found, which demonstrates the linearity of our system and confirms that our system gives accurate results that compare quite well to the TruCount method. The difference of 17% between the two methods is not unusual for
comparing two methods (21). The error bar represents the standard deviation of 5 replicates of CD4⁺ T lymphocyte counts obtained by the SC 3.0 system.

FIG. 9. Linearity and accuracy of SC3.0 system within the clinically important range of CD4⁺ T lymphocytes counts (0 ~ 600/µl). The error bar represents the standard deviation of 5 replicates of CD4⁺ T lymphocyte counts obtained by the SC3.0 system.

CD4 Enumeration of HIV⁺ Patients

We tested our activation method by measuring blood specimens of 42 HIV⁺ patients. The CD4 counts obtained by SC 3.0 were compared with those obtained by SP FCM tetraCXP method. (CD4 counts range from 65 to 1403/µl, by FCM). Figure 10 shows the linear regression (A) and the Bland-Altman plot (B) of the CD4 counts obtained by these 2 methods. Linear regression shows a reasonably good slope and correlation coefficient (SC 3.0 = 1.03 × SP FCM, R = 0.93). Bland-Altman plot shows a bias of 10.6%, with the limits of agreement ranging from -22.9% to 44.2%.
Antibodies determine usually an important part of the price of tests for counting CD4\(^+\) T lymphocytes in HIV infected patients and it is therefore preferred to use as few antibodies as possible. However, interference of CD4\(^{dim}\) monocytes in CD4 enumeration methods, when using only CD4 antibodies for cell recognition, is a well known problem in HIV staging (22, 23). Our method circumvents this challenge by applying CD4 immuno-magnetic selection of the target cells in combination with a discrimination of co-collected granulocytes and monocytes. To discriminate the false positive cells, we stimulate an oxidative burst in these co-collected cells and count these cells using the R123 fluorescence that evolves as a result of the oxidation of DHR123 to R123; ROS produced by the oxidative burst is responsible for the oxidation. From our results we conclude that the method works fairly well.

In clinics, the oxidative burst activity is mainly tested to diagnose inborn defects, such as chronic granulomatous disease (CGD) (24). To the best of our knowledge, this is the first time that the oxidative burst is applied to enumerate CD4 cells by distinguishing phagocytes (CD4\(^{dim}\) monocytes and non-specifically adhered granulocytes, mainly neutrophils) from CD4\(^+\) T lymphocytes. Bursttest Kit from Orpegen Pharma has been developed to determine the oxidative burst activity of monocytes and granulocytes in
heparinized whole blood using FCM. However, the sample preparation using Bursttest Kit is very complicated (25). In our method, only 15 min of reagents cocktail incubation in dark at RT is needed. Separate oxidation, lysing, fixation, washing, and DNA staining steps, and the usage of ice bath and of 37 °C water bath are avoided. In comparison, our method is much simpler. Most importantly, we could implement the stimulation and detection of the oxidative burst into our immuno-magnetic labeling method, and obtained a novel method for enumeration of CD4^+ T lymphocytes.

As our CD4 enumeration system is meant to be applied in a tropical climate, the effect of tropical temperatures on the oxidative burst activity was investigated. We noticed that at 37 °C, the cell activation is faster than at RT, and a brighter R123 image is recorded for both monocytes and neutrophils. It suggests that the oxidative burst activity is higher at 37 °C than at RT. In regions with high temperatures, the reagents incubation time may be shorter; and the brighter R123 intensity can further facilitate the distinction of monocytes and granulocytes from lymphocytes.

We have tried to increase the R123 intensity evoked in activated monocytes. It was reported that granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor-α (TNF-α) rapidly (within 10 min) primed suspended human monocytes and enhanced O_{2^-} release stimulated by N-Formyl-Met-Leu-Phe (FMLP). FMLP is a potent chemotactic peptide, which induces a metabolic burst in phagocytes accompanied by an increase in respiratory rate. The priming effect by the combination of optimal concentrations of TNF-α and GM-CSF to monocytes was greater than that by each cytokine alone (26, 27). We investigated whether pre-incubation (10 min or 30 min at 37 °C) with GM-CSF (5 ng/ml, 50 ng/ml or 500 ng/ml) and/or TNF-α (0.5 ng/ml, 5 ng/ml, 50 ng/ml and 500 ng/ml) at 37°C could prime the oxidative burst of monocytes induced by FMLP in whole blood. However, there was no evidence of such a priming effect. The reason why our experiments showed different results from those reported elsewhere (26) is not clear. The experiment of this article was performed on the isolated monocytes suspension. The isolation of monocytes from their whole blood environment by various procedures may probably modulate surface receptor expression in a different manner and alter cell response. We concluded that in our system, the oxidative burst of monocytes
stimulated by PMA could not be boosted by priming the cells with GM-CSF and/or TNF-α upon FMLP stimulation.

In our study, we did not specifically look at the oxidative burst level of eosinophils, as it is well known that eosinophils produce more superoxide anions (O₂⁻) than neutrophils (28, 29). Any non-specifically attracted eosinophils on the analysis surface will be recorded and counted in the R123 images.

FIG. 11. The platelets aggregation and platelets-leukocytes adhesion in Heparin blood (A) but not in EDTA blood (B). Cells are in bright R123 staining and platelets aggregation is in very dim R123 staining.

To investigate which blood anti-coagulant is better for preserving the metabolic activity of cells, blood samples drawn in EDTA tubes and samples drawn in heparin tubes were compared (Figure 11). We observed that in the sample prepared using heparin-anticoagulated whole blood, platelets aggregated and to a great extent adhered to monocytes and granulocytes in agreement with literature (30 - 32). By PMA stimulation, platelets could probably be even more active. These cell aggregations make a reliable cell enumeration impossible. (In the commercial kit, these aggregations are depleted by the lysing and washing steps.) No aggregations were found in EDTA blood. However, on the second day after blood draw, part of the monocytes and neutrophils were inactive or dying, showing a low R123 intensity. We conclude that heparin cannot
be used in our system, and that EDTA blood should be tested as soon as possible and no longer than 12 hours after blood draw.

Our system is not applicable for CD4 enumeration of samples of patients with CGD disease due to the reduced or missing oxidative burst activity for cells of these patients (24). Given the very low incidence of CGD this will probably not be a major problem for application of our test.

Shalekoff et al. reported a depressed oxidative burst in polymorphonuclear leukocytes from individuals with pulmonary tuberculosis with or without HIV-1 infection (33), and Dobmeyer et al. reported that the decreased function of monocytes and granulocytes during HIV-1 infection correlates with CD4 cell counts (34). In our test on HIV\(^+\) patients, the oxidative burst of cells from HIV\(^+\) patients with low CD4 counts (50 – 200/\(\mu\)l) is still detectable. We did not see the depression in oxidative burst level for these patients. To draw further conclusions we will have to field-test our system in resource-constrained countries on AIDS- and HIV\(^+\) patients with pulmonary tuberculosis.

We noticed that for 30 – 40\% of the HIV\(^+\) patients the Hoechst33342 image showed big (20 – 200 \(\mu\)m) Hoechst33342 stained objects, which varies in morphology. These are probably DNA fragments stained by Hoechst33342. The accuracy of cell enumeration by applying image analysis was sometimes hampered by these objects. We are currently searching for methods to prevent such clumps. Currently we use our image analysis program to discriminate the clumps in counting.

In our SC 3.0 system, sample preparation is very simple: only a few pipetting steps are needed, which is therefore the most essential part in training the operators. The instrument is also easy-to-handle, even for less trained operators. Two pairs of LEDs, for excitation of Hoechst33342 and R123, are manually switched, and two emission filters are manually changed by sliding a filter changer. Image recording and analysis only takes 2 minutes. Data interpretation is performed by the computer, which prevents subjective errors.

Our instrument costs about US$ 3,000 (single unit component price). Eventually mass production may bring this price to a much lower level. Since only CD4 antibody is applied, the price per test is low (about US$ 0.9 for reagents and US$ 1.8 for the...
A Novel Affordable CD4 Enumeration

A disposable sample chamber). Currently we are developing a cheaper chamber to reduce the price per test. Moreover, the system is portable, robust, battery-operated, and suited for point-of-care applications. It can be operated in extremely remote areas, immediately after arrival on site, and if needed from a car battery.

In conclusion, this novel affordable CD4 enumeration system may be a good candidate for CD4\(^+\) T lymphocytes enumeration in point-of-care settings in resource-constrained countries.

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CHAPTER 7

Evaluation of an Easy-to-use Image Cytometer for CD4 and CD8 Enumeration on HIV Infected Patients in Thailand

ABSTRACT

Background: We developed a volumetric single platform image cytometer (SP ICM) that counts immuno-magnetically selected CD3⁺ T lymphocytes and fluorescently labeled CD4⁺ T and CD8⁺ T lymphocytes. The battery operated instrument was designed to be low-cost, yet reliable and robust. The immuno-labeling methods and the instrumentation are easy-to-use even for less trained operators. In this study, we evaluated the performance of this system on HIV infected patients in Thailand.

Methods: CD4, CD8 counts and CD4/CD8 ratios in whole blood samples of 460 HIV⁺ adults and 47 HIV⁺ children were analyzed by SP ICM, and results were compared with those obtained by FACSCount, a single platform flow cytometer (SP FCM), and by FACSCan, a dual platform (DP) FCM.

Results: CD4 counts by SP ICM were comparable ($R \geq 0.97$) with those by the FACSCount and the FACSCan with a bias of 9.1% and 11.2%, respectively. At very low CD4 counts ($\leq 50/\mu l$), some over-count outliers were observed by the FACSCan and SP ICM when compared to the FACSCount. For CD8 enumeration, the SP ICM showed a good correlation ($R \geq 0.95$), and a consistent undercount (~ 15%) as compared to the FACSCount.

Conclusions: Evaluation of the SP ICM for CD4 and CD8 enumeration demonstrated comparable results with FCM in a population of HIV infected patients. The SP ICM is a good alternative method for point-of-care settings in resource-constrained countries.

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INTRODUCTION

It is estimated that in 2005, 40.3 million human beings were living with HIV, of whom approximately 95% are in the developing countries (1). Recently, inexpensive and generic antiretroviral treatment (ART) has become available to more and more patients in middle- and low-income countries, such as Thailand. In Thailand, more than 1 million people are infected with HIV, and about 30,000 new infections occur annually (2). The availability of affordable and reliable CD4 enumeration for the initiation and monitoring of ART has become a critical issue.

Single platform (SP) and dual platform (DP) flow cytometry (FCM) methods are currently the most accepted methods for CD4\(^+\) T lymphocytes enumeration. The SP FCM methods use calibration beads (3) or employ a volumetric method (4). The DP FCM methods calculate the absolute CD4 count by multiplying the lymphocytes count obtained from an automatic hematology analyzer with the CD4 percentage obtained from FCM (5). The DP FCM methods are usually less accurate as compared with the SP FCM methods because of variation among hematology analyzers (6). Both SP and DP methods are expensive in equipment, maintenance, technician training and assay cost (7).

Although dedicated CD4 FCM systems (BD FACSCount, Guava Easy CD4 and Partec CyFlow) are less expensive, the costs of the instruments and the assays are not yet affordable for resource-constrained countries. Furthermore, the operation and maintenance of a FCM requires well-trained technicians and stable electricity (7 - 9). Development of an affordable, simple, easy-to-use and reliable CD4\(^+\) T lymphocytes enumeration system is therefore urgently needed.

We developed a single platform image cytometer (SP ICM) system, StarCount 2.1, which is a volumetric CD4\(^+\) T and CD8\(^+\) T lymphocytes enumeration and CD4/CD8 ratio determination system (10 - 11). In this system, CD3 immuno-magnetic selection is combined with immuno-fluorescent labeling of CD4-Phycoerythrin (PE) and CD8-Peridinin-Chlorophyll-Protein (PerCP). After CD3 cells in whole blood are immuno-magnetically attracted to an analysis surface, the fluorescent images of the CD4\(^+\) T lymphocytes and CD8\(^+\) T lymphocytes are captured and the individual cells are identified and counted. CD4/CD8 ratio is determined accordingly. The instrument was
designed to be low-cost (components cost US$ 3,000), compact (25 cm × 25 cm × 20 cm), yet reliable and easy-to-use. It is computer-controlled, and operates on a 12 V rechargeable battery.

In this study, we evaluate the performance of the system in HIV infected patients in Thailand. CD4, CD8 counts and CD4/CD8 ratios in whole blood samples of 460 HIV+ adults and 47 HIV+ children obtained by the SP ICM were compared with the results obtained by a SP FCM FACSCount and by a DP FCM FACSCan. The FACSCount is a dedicated SP FCM for CD4 and CD8 T cell counting and has been extensively validated and widely used in many resource-poor settings and is considered the gold standard (12 - 14). The FACSCount instrument costs around US$ 30,000 and one test costs ~ US$ 15. The DP FCM is the accepted standard method for CD4 enumeration in Thailand (5).

MATERIALS AND METHODS

Patients and Blood Samples

Blood specimens from 507 HIV infected patients were supplied by Siriraj Hospital, Bangkok, Thailand. The patients consisted of 460 adults (215 male and 245 female, ranging in age from 18 to 75 years (mean 40 ± 10)), and 47 children (27 male and 20 female, ranging in age from 4 to 15 years (mean 9.3 ± 3.0)). Thirteen of the adult patients were co-infected with tuberculosis (TB). All blood specimens were collected in sterile K3EDTA blood collection tubes by venipuncture and processed within 10 hrs after draw. The specimens were leftover clinical specimens that were anonymous at the Department of Immunology, Faculty of Medicine, Siriraj Hospital. This study was approved by the Ethics Committee of the Faculty of Medicine, Siriraj Hospital, Mahdol University.

Methods

SP image cytometer method (SP ICM)

The SP ICM is a dedicated volumetric CD4+ T and CD8+ T lymphocytes enumeration system for HIV monitoring (10 - 11).

Sample preparation was performed in the following way: To 100 μl (25 μl was used for children’s blood sample) whole blood, 10 μl reagent cocktail, which contains 3 μl of
0.655 mg/ml CD3-FerroFluid (CD3FF, clone: CRIS-7, isotype: mouse IgG2a-κ, Immunicon Inc., USA), 1 μl of 12.5 μg/ml CD4PE (clone: RPA-T4, isotype: Mouse IgG1-κ, BD Pharmingen, USA), 3 μl of 6.25 μg/ml CD8PerCP (clone: SK1, isotype: Mouse IgG1-κ, BD, USA) and 3 μl of system buffer (Immunicon Inc., USA) was added and mixed. After 15 min incubation, the sample was diluted with 290 μl (365 μl for children’s blood sample) of system buffer to a final volume of 400 μl. Approximately 340 μl of the sample solution was transferred into the analysis chamber. The chamber was plugged and placed into a magnet assembly (MagNest®; Immunicon Inc., USA). After 20 min magnetic separation, the sample was ready to be analyzed.

To obtain absolute cell counts, the CD4PE and CD8PerCP fluorescence images were captured from three different positions using the camera software (CCDOPS, SBIG, USA). At each position the PE and PerCP filters were manually changed. Each image captured from separate positions corresponds to 1.16 μl of whole blood. The three CD4 and three CD8 images are captured and stored in approximately 3 min. The total volume (3.48 μl) was chosen to obtain a theoretical statistical Poisson variation of approximately 5.4% at 100 cells/μl and approximately 7.6% at 50 cells/μl (10).

Dedicated image analysis algorithms were written in ImageJ, a public-domain Java image processing software package (NIH, Maryland, USA) (15). For this evaluation the sample images were saved after the measurements to permit the comparisons of different algorithms on a PC. Detailed explanation of the different algorithms and the results obtained are provided elsewhere (chapter 5). The algorithm that showed the best performance in a test was used to analyze the data from this study.


To evaluate the analysis speed of this algorithm the program was run on the single board computer (665 MHz processor with 256 MB RAM) present in the image cytometer. The algorithm needs approximately 120 ~ 180 seconds to analyze three CD4 and three CD8 images when the number of cells per image is in the range of 58 ~ 1393, i.e., 50 ~ 1200 cells/μl.
SP FCM FACSCount method (FACSCount)

The FACSCount system consist of a bench-top FCM equipped with a green laser, a twin-tube containing calibration beads and CD4PE/CD3PE-Cyochrome5 (PE-Cy5) and CD8PE/CD3PE-Cy5, respectively, controls and software. CD3⁺ T lymphocytes, CD4⁺ T and CD8⁺ T lymphocytes count and CD4/CD8 ratio are provided. Sample preparation and acquisition was performed according to the manufacturer’s recommendation (BD Biosciences, USA) (16). For children samples, whole blood was diluted 4 times with PBS.

DP FCM FACSCan method (FACSCan)

CD4 count was obtained using FACSCan (BD Biosciences, USA) and a hematology analyzer (Sysmex XT2000, TOA Medical Electronics, Kobe, Japan). The absolute number of lymphocytes was determined by a hematology analyzer. The percentage of CD4⁺ T lymphocytes from total lymphocytes were obtained by FACSCan using CellQuest software. This test was performed by the routine clinical laboratory of the Siriraj Hospital, Bangkok, Thailand.

Statistical Analysis

Based on the CD4⁺ T, CD8⁺ T lymphocytes counts and CD4/CD8 ratio obtained from different methods, linear regression lines were drawn and correlation coefficients (R) were calculated. Bland-Altman plots (17, 18) were used to evaluate the interchange-ability between methods. In Bland-Altman plots, the average of the CD4 or CD8 counts or CD4/CD8 ratio obtained from two methods is plotted on the horizontal axis, and the difference/average (%) is plotted on the vertical axis. The solid line in the plot represents the bias (the average difference between the two methods), and the dashed lines in the plot illustrate the upper and lower limits of agreement (± 1.96 SD).

To examine possible differences of the CD4 counts obtained by these methods in the most significant clinical range for HIV⁺ adults, statistical analysis of the blood specimens from HIV⁺ adults with low CD4 count (≤ 200/μl) and with CD4 counts higher than 200/μl was performed separately. For blood samples of HIV⁺ adults with very low CD4 count (≤
50/µl, the difference of absolute CD4 counts is plotted on the vertical axis of the Bland-Altman plot.

RESULTS

CD4 Enumeration

Blood specimens of HIV+ patients were tested by the SP ICM, the FACSCount and the FACSCan, respectively. The obtained CD4+ T lymphocytes counts were compared with each other. Comparisons of CD4 counts of adults, children, and all HIV+ patients were performed separately. Slopes and correlation coefficients (R) from the linear regression plots and biases (%) and limits of agreement (%) from the Bland-Altman plots were summarized in Table 1.

Table 1. Comparison of CD4+ T lymphocytes counts obtained by the SP ICM, the FACSCount and the DP FCM FACSCan. The table shows the slopes and correlation coefficients (R) from the linear regression plots, and biases (%) and limits of agreement (%) (LL: lower limit; UL: upper limit) from the Bland-Altman plots of CD4 counts of adults, children, and all HIV+ patients.

<table>
<thead>
<tr>
<th>FACSCan vs FACSCount</th>
<th>SP ICM vs FACSCount</th>
<th>SP ICM vs FACSCan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults (435)</td>
<td>Adults (460, 13TB)</td>
<td>Adults (437)</td>
</tr>
<tr>
<td>Slope 0.92 Bias -2.3</td>
<td>Slope 1.04 Bias 7.3</td>
<td>Slope 1.10 Bias 9.1</td>
</tr>
<tr>
<td>R 0.98 LL -42.2</td>
<td>R 0.99 LL -33.7</td>
<td>R 0.97 LL -24.9</td>
</tr>
<tr>
<td>UL 37.6</td>
<td>UL 48.2</td>
<td>UL 43.0</td>
</tr>
<tr>
<td>Children (47)</td>
<td>Children (47)</td>
<td>Children (47)</td>
</tr>
<tr>
<td>Slope 0.93 Bias -3.8</td>
<td>Slope 1.13 Bias 24.8</td>
<td>Slope 1.23 Bias 29.0</td>
</tr>
<tr>
<td>R 0.99 LL -47.7</td>
<td>R 0.99 LL -42.0</td>
<td>R 0.98 LL -23.3</td>
</tr>
<tr>
<td>UL 40.1</td>
<td>UL 91.6</td>
<td>UL 81.3</td>
</tr>
<tr>
<td>All patients (482)</td>
<td>All patients (507)</td>
<td>All patients (484)</td>
</tr>
<tr>
<td>Slope 0.92 Bias -2.5</td>
<td>Slope 1.06 Bias 9.1</td>
<td>Slope 1.14 Bias 11.2</td>
</tr>
<tr>
<td>R 0.98 LL -42.7</td>
<td>R 0.99 LL -34.5</td>
<td>R 0.98 LL -26.3</td>
</tr>
<tr>
<td>UL 37.8</td>
<td>UL 52.6</td>
<td>UL 48.7</td>
</tr>
</tbody>
</table>

First, the FACSCan was compared with the FACSCount. Excellent correlations were obtained (R ≥ 0.98; Slope: 0.92 for the adults; 0.93 for the children; and 0.92 for all patients). Bland-Altman plots showed the interchangeability between two FCM methods.
with small biases (Bias: -2.3% for the adults; -3.8% for the children; and -2.5% for all patients).

Secondly, the SP ICM was compared with the FACSCount. Excellent correlations were obtained as well ($R = 0.99$; Slope: 1.04 for adults; 1.15 for children; and 1.06 for all patients). Bland-Altman plots showed the interchange-ability between two methods with certain biases (Bias: 7.3% for adults; 24.8% for children; and 9.1% for all patients).

Thirdly, the SP ICM was compared with the FACSCan. Good correlations were obtained ($R = 0.97$ - 0.98; Slope: 1.10 for adults; 1.23 for children; and 1.14 for all patients). Bland-Altman plots showed the interchange-ability between two methods with certain biases (Bias: 9.1% for the adults; 29.0% for the children; and 11.2% for all patients).

To examine the possible differences of the CD4 counts obtained by these methods in the clinical significant range of CD4 counts of $\leq 200/\mu l$ for HIV$^+$ adults, statistical analysis of all blood specimens, blood specimens with low CD4 count ($\leq 200/\mu l$) and CD4 counts of more than 200/μl was performed separately. Slopes and correlation coefficients ($R$) from the linear regression plots and biases (%) and limits of agreement (%) from the Bland-Altman plots were summarized in Table 2. Also the Bland-Altman plots comparing CD4 counts of HIV$^+$ adults between the different methods were shown in Figure 1. Analysis of all samples (A), samples with low CD4 counts ($\leq 200/\mu l$) (B), and samples with very low CD4 counts ($\leq 50/\mu l$) (C), were performed separately. It should be noted that in the very low CD4 count region ($\leq 50/\mu l$), outliers happened in all three comparisons.
FIG. 1. Bland-Altman plots of comparisons of CD4 counts of HIV+ adult patients between different methods. Analysis of all samples (A), samples with low CD4 counts (≤ 200/µl) (B), and samples with very low CD4 counts (≤ 50/µl) (C), were performed separately. A1 (435 adults), B1 (92 adults), and C1 (26 adults): the FACSCan versus the FACSCount; A2 (460 adults), B2 (106 adults), and C2 (29 adults): the SP ICM versus the FACSCount; A3 (437 adults), B3 (97 adults), and C3 (27 adults): the SP ICM versus the FACSCan.
Table 2. Comparison of CD4+ T lymphocytes counts obtained by the SP ICM, the FACSCount and the FACSCan for HIV+ adults in the low CD4 count range (≤ 200/μl) and the range with more than 200/μl (> 200/μl). The table shows the slopes and correlation coefficients ($R$) from the linear regression plots, and biases (%) and limits of agreement (%) (LL: lower limit; UL: upper limit) from the Bland-Altman plots.

<table>
<thead>
<tr>
<th>Range</th>
<th>FACSCan vs FACSCount</th>
<th>SP ICM vs FACSCount</th>
<th>SP ICM vs FACSCan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults</td>
<td>#</td>
<td>all</td>
<td>≤ 200/μl</td>
</tr>
<tr>
<td>Slope</td>
<td>0.92</td>
<td>0.97</td>
<td>0.92</td>
</tr>
<tr>
<td>$R$</td>
<td>0.98</td>
<td>0.98</td>
<td>0.97</td>
</tr>
<tr>
<td>Bias</td>
<td>-2.3</td>
<td>13.4</td>
<td>-6.5</td>
</tr>
<tr>
<td>LL</td>
<td>-42.2</td>
<td>-56.0</td>
<td>-26.8</td>
</tr>
<tr>
<td>UL</td>
<td>37.6</td>
<td>82.8</td>
<td>13.7</td>
</tr>
</tbody>
</table>

The linear regression plots of these three comparisons in the CD4 count range of more than 200/μl followed the same tendency as the plots in the whole CD4 counts range.

The Bland-Altman plots of these three comparisons in the CD4 count range of more than 200/μl showed the ability to interchange the different methods. For the comparison between the FACSCan and the FACSCount, the bias of -6.5% indicated that CD4 count by the FACSCan was consistently about 6.5% less than that by FACSCount, which agreed well with the slope of 0.92 for adults in the CD4 count range of more than 200/μl.

The increase in the bias between the FACSCan and the FACSCount can be attributed to the over-count outliers for the very low CD4 counts (bias: -2.3% for all, -6.5% for > 200/μl, and 13.4% for < 200/μl).

For the comparison between the SP ICM and the FACSCount, the bias (3.5%) indicated that the two methods were interchangeable, which also agreed well with the slope of 1.04 for adults in the CD4 count range of more than 200/μl. The bias between the SP ICM and the FACSCount increased from 3.5% (> 200/μl) to 7.3% (the whole range) due to the over-count outliers observed for some of the very low CD4 counts.
For the comparison between the SP ICM and the FACSCan, the bias indicated that CD4 count by the SP ICM was consistently about 10% higher than that of the FACSCan. And the biases almost kept same (9.8% for > 200/μl; and 9.1% for the whole range).

For the low CD4 count (≤ 200/μl), good correlations (R = 0.97 - 0.98) were still achieved. However, for the comparisons between the FACSCan and the FACSCount and between the SP ICM and the FACSCount, the biases of the Bland-Altman plots were dramatically raised due to the over-count outliers at the very low CD4 counts (≤ 50/μl). Also the limits of agreement ranges of three comparisons were increased dramatically as compared to those of samples with CD4 count more than 200/μl (Table 2).

For samples with very low CD4 count (≤ 50/μl), the Bland Altman analyses were performed in a different way: the difference of absolute CD4 counts was plotted on the vertical axis (Figure 1C). Results indicated that on average the FACSCan counted 7 cells/μl (limits of agreement: -7 – 21 cells/μl) more than the FACSCount did; the SP ICM counted 6 cells/μl (limits of agreement: -12 – 25 cells/μl) more than the FACSCount did. And the bias between the SP ICM and the FACSCan is only 1 cells/μl (limits of agreement: -21 – 23 cells/μl).

**CD8 Enumeration**

By the SP ICM and the FACSCount methods, CD8 counts were obtained as well as CD4 counts. Thirty-four of the 507 HIV+ patient samples were excluded from the comparison for CD8 enumeration between the SP ICM and the FACSCount: Thirty-three adult HIV+ samples were excludes because the FACSCount reports all CD8+ T lymphocytes counts more than 2000/μl as greater than 2000; one sample of a HIV+ child was not counted by the FACSCount. Figure 2 shows the linear regression plots (A) and Bland-Altman plots (B) comparing CD8 counts obtained by the SP ICM and the FACSCount for 427 adults, 46 children, and all 473 HIV+ patients. The results were summarized in Table 3.
FIG. 2. Linear regression plots (A) and Bland-Altman plots (B) comparing CD8 counts obtained by the SP ICM and the FACSCount for 427 adults (A1, B1), 46 children (A2, B2), and all 473 HIV+ patients (A3, B3).
Table 3. Comparison of CD8+ T lymphocytes counts and the CD4/CD8 ratio by the SP ICM and the FACSCount. The table shows the slopes and correlation coefficients ($R$) from the linear regression plots, and biases and limits of agreement (LL: lower limit; UL: upper limit) from the Bland-Altman plots of adults (427), children (46), and of all HIV+ patients (473).

<table>
<thead>
<tr>
<th></th>
<th>CD8</th>
<th>CD4/CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adults (427, 13TB)</td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.83</td>
<td>1.22</td>
</tr>
<tr>
<td>Bias</td>
<td>-16.8</td>
<td>23.5</td>
</tr>
<tr>
<td>$R$</td>
<td>0.95</td>
<td>0.99</td>
</tr>
<tr>
<td>LL</td>
<td>-42.5</td>
<td>-9.4</td>
</tr>
<tr>
<td>UL</td>
<td>8.8</td>
<td>56.4</td>
</tr>
<tr>
<td>Children (46)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.97</td>
<td>1.18</td>
</tr>
<tr>
<td>Bias</td>
<td>-3.6</td>
<td>25.7</td>
</tr>
<tr>
<td>$R$</td>
<td>0.99</td>
<td>0.98</td>
</tr>
<tr>
<td>LL</td>
<td>-19.7</td>
<td>-22.0</td>
</tr>
<tr>
<td>UL</td>
<td>12.5</td>
<td>73.4</td>
</tr>
<tr>
<td>All patients (473)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.86</td>
<td>1.22</td>
</tr>
<tr>
<td>Bias</td>
<td>-15.6</td>
<td>23.7</td>
</tr>
<tr>
<td>$R$</td>
<td>0.96</td>
<td>0.99</td>
</tr>
<tr>
<td>LL</td>
<td>-41.6</td>
<td>-10.8</td>
</tr>
<tr>
<td>UL</td>
<td>10.4</td>
<td>58.3</td>
</tr>
</tbody>
</table>

For CD8 enumeration, good correlations were obtained ($R = 0.95 - 0.99$; Slope: 0.83 for adults; 0.97 for children; and 0.86 for all patients). Bland-Altman plot shows the interchange-ability between two methods with certain biases (Bias: -16.8% for adults; -3.6% for children; and -15.6% for all patients).

CD4/CD8 Ratio Determination

The CD4/CD8 ratios were determined by dividing the CD4 count by the CD8 count. The comparisons were performed between the SP ICM and the FACSCount and the results were also summarized in Table 3. Excellent correlations were achieved ($R \geq 0.98$; Slope: 1.22 for adults; 1.18 for children; and 1.22 for all patients). Bland-Altman plot shows the interchange-ability between two methods with certain biases (Bias: 23.5% for adults; 25.7% for children; and 23.7% for all patients). As the CD8 counts were somewhat lower in the SP ICM compared to the FACSCount, whereas the CD4 counts were somewhat higher, CD4/CD8 ratios are ~ 24% higher in the SP ICM as compared to the FACSCount.
Over-count Outliers

To investigate the cause of the over-count outliers in the very low CD4 count range, the images of those over-count outliers were studied. Figure 3 shows an example of CD4PE images of a CD4 over-count outlier counted by the image analysis algorithm (A), by a manually selection of the cells in the image (B); and an image of a normal sample counted by the image analysis algorithm (C). The objects with red circles were counted by the image analysis algorithm, while those in red boxes were manually selected by a trained operator according to their nice round shape, bright fluorescence and regular size.

For the outlier image, 13 cells were counted by the image analysis algorithm (Fig 3A). By manually selecting, only 8 objects in red boxes were counted (Fig 3C), which is close to the CD4 count by the FACSCount. It was found that in the SP ICM method, the image quality of samples with very low CD4 counts (Fig 3A, 3B) is much poorer than that of samples with more than 50 CD4 counts per μl (Fig 3C), in terms of high and noisy background, uneven cell intensities and presence of more cell debris. The image analysis algorithm could not distinguish CD4⁺ T lymphocytes clearly from cell debris, clumping of fluorophores, auto-fluorescent junks, and background noise in some images with very low CD4 counts. For a sample with more than 50 CD4⁺ T lymphocytes per μl, the image is much better and the image algorithm can count CD4⁺ T lymphocytes accurately (Fig 3C). This is the main cause of the over-count outliers in the very low CD4 count range in the SP ICM.
FIG. 3. CD4PE images of a CD4 over-count outlier (FACSCount: 5/µl) (A, B) and a normal sample (FACSCount: 518/µl) (C). A: 13 cells were selected by the image analysis algorithm and are indicated by the red circles (10/µl); B: 8 cells were manually selected by a trained operator and are indicated by the red boxes (7/µl); C: CD4+ T lymphocytes in an image of a normal sample were counted by the image algorithm and indicated by the red circles (543/µl). The small black events in the images are red blood cells adhered to the glass. The green arrows indicate events that were counted by the image analysis algorithm, but not selected as intact cells by the
Evaluation of Image Cytometer in Thailand

trained operator, as they were believed to be either too small or did not have a sufficiently round shape and assumed to be cell debris or clumping of fluorophores. The difference in intensity of the images in A and B are due to the auto-scaling. (color figures on Page 216)

DISCUSSION

CD4 counts by the SP ICM were comparable with those by the SP FCM FACSCount method and the DP FCM FACSCan method. Across the CD4 count range, the SP ICM counted ~9.1% more as compared to the FACSCount and ~11.2% more as compared to the FACSCan. Previous reports comparing CD4 counts between laboratories reported coefficients of variation (CVs) of 10 ~ 18.3 % for SP FCM systems and of 14.5 ~ 43.7 % for DP FCM systems (19, 20). When completely different methods and instruments are compared, the systematic error may be even higher. The biases between our SP ICM and the FACSCount and the FACSCan are within the reported ranges.

At very low CD4 counts (1 ~ 50/μl), some outliers were observed in the FACSCan and the SP ICM when compared to the FACSCount. The FACSCount was considered the gold standard (6, 12 - 14). The FACSCan and the SP ICM counted ~7 cells/μl and 6 cells/μl more than the FACSCount did, respectively. Nevertheless, the biases in this range may not influence the clinical decision making, because ART are given when CD4 counts are less than 200/μl (21). These biases will unlikely affect the monitoring either, as long as the same method was used all the time.

The most likely explanation for the over-count outliers is the increasing contribution of cell debris, fluorophore clumps and auto-fluorescent junk in the images with lower cell counts. This can lead to the misclassification of events by the algorithm. Evaluation of different methods to detect cells in the images captured by the SP ICM showed a clear difference in performance especially at the low and high cell counts. Review of the images of the outliers indeed showed that some had a noisy background. In FCM, some of these issues are avoided by the use of forward-and side-scatter and quantitative analysis of the fluorescence signals. Further development of the image analysis algorithms used in the image cytometer can improve the discrimination of
intact target cells from debris and reduce or eliminate the outliers. Another explanation for the outliers observed between methods in the very low CD4 count (< 50/μl) may be contributed to the higher variation of the SP FCM FACSCount method (CV > 10% at CD4 counts below 50/μl) (16).

The possible explanations for the CD8 undercount (~ 15%) by the SP ICM as compared to the FACSCount had been discussed extensively elsewhere (chapter 5). Part of the reason was due to the CD8dim T lymphocytes (about 10% of total CD8+ T lymphocytes). They were either not detected by the SP ICM due to the limited sensitivity of the instrument or not counted by the image analysis due to the interference of the cross-talked CD4PE cells in CD8PerCP image. Similarly, the CD8 undercount was also found in some dedicated CD4 and CD8 numeration SP FCM methods, such as Guava PCA method (13), due to its limited sensitivity and gate setting by which only the CD8bright T lymphocytes are counted.

A method to prevent the interference of the cross-talk of CD4PE cells in the CD8PerCP image is to prepare CD4 and CD8 enumeration samples in two separate analysis chambers. This however would double the test cost and sample preparation time. Another alternative is to improve the algorithm to discriminate the CD8dim T lymphocytes that are not present in the CD4 image from the CD4 PE cells that appear in the CD4 as well as CD8 image.

It was found in this study that CD4 as well as CD8 counts of HIV+ children are ~10% higher than those of the HIV+ adult patients obtained by the SP ICM, as shown in Table 1 and Table 3. Potential reasons for this phenomenon are: 1. T lymphocytes from elderly individuals express lower numbers of CD3 receptors ((61 ± 10) x 10^3) as compared to young individuals ((69 ± 10) x 10^3) in healthy populations (22). 2. The numbers of receptors for CD3, CD4 and CD8 are significantly decreased on T lymphocytes from HIV infected patients (23). The extent of CD3 receptor loss in HIV+ adults is probably larger than that in HIV+ children. Some T lymphocytes with less CD3 receptors may not be magnetically attracted to the surface within the 20 min magnetic separation. Adding more CD3FF and longer magnetic separation time may help.
Our SP ICM is simple and easy-to-handle, even for less trained operators. No data interpretation is needed, which prevents subjective errors. Acquiring pipetting skills for the 3 pipetting steps in sample preparation is the most essential part in training the operators. Moreover, the system is portable, robust, battery-operated, and suitable for point-of-care applications. It can be operated in remote areas, and can run on a car battery. Due to the simplicity and the fixed position of the optical components, no alignment or calibration is required. Different from FCM, no cleaning step is needed, which make it very convenient for the users. This is especially advantageous for usage in developing countries.

In conclusion, the SP ICM can be a good alternative method for the SP and DP FCM methods in point-of-care settings of resource-constrained countries.

ACKNOWLEDGEMENTS
This study is financially supported by STW, the Dutch Technology Foundation (project TGT, 6146). We thank Mr. Charin Thepthai from the routine Lab in the Siriraj Hospital, Bangkok, Thailand for kindly supplying fresh blood samples and the CD4 counts by the DP FCM FACScan method. We acknowledge all patients and healthy donors whose blood was tested in this study.

REFERENCES


A CD3 Cell Immobilization Platform for CD4 and CD8 Enumeration

ABSTRACT

Background: The urgent need for affordable CD4 enumeration facilities for monitoring HIV infection stage has been stimulating the development of new point-of-care CD4 enumeration technologies.

Methods: In this chapter, we present a novel technology of building up a CD3+ T lymphocytes immobilization platform for CD4 and CD8 enumeration. A technology to build up complex bio-structures consisting of a basic system was applied, in which streptavidin (SAV) is bound to β-Cyclodextrin (βCD) self-assembled monolayers (SAMs) on silicon oxide surface. CD3 monoclonal antibodies (MAbs) were attached to the βCD SAMs via the SAV to form a CD3 cell immobilization platform.

Results: Dot blot experiments indicated that biotin-protein G (biotin-PG) is suitable as an Fc receptor for the CD3 Monoclonal Antibody (MAb) (B-B12 or biotin-CRIS-7) to build up the CD3 MAb layers. Surface Plasmon Resonance (SPR) sensograms showed a successful stepwise build-up of a biotin-CRIS-7 layer and B-B12 layer on the βCD SAMs in two different schemes. A preliminary cell experiment was performed to investigate the ability of the B-B12 layer of capturing the CD3+ T lymphocytes. The CD3+ T lymphocytes, the CD3+CD4+ T lymphocytes and the CD3+CD8+ T lymphocytes were enumerated and the relationship between the input cells and output captured cells was studied.

The content of this chapter is a part of a manuscript submitted for publication. Ludden MJW, Li X, Greve J, van Amerongen A, Escalante M, Subramanian V, Reinhout DN, Huskens J. Assembly of bionanostructures consisting of antibodies at β-Cyclodextrin molecular printboards. equally contributed authorship.
Conclusions: We propose that a novel CD4 enumeration method can be developed based on the technology of buildup of bio-structures at the [CD SAMs and on a simple and low-cost, single-platform image cytometer developed earlier in our group.

INTRODUCTION

Since the first official documentation of the AIDS pandemic in 1981 (1), AIDS has developed into a global crisis that forms an immediate emergency and a long-term development issue. For adults, the CD4$^+$ T lymphocytes count is essential to decide when to start the antiretroviral treatment, to monitor the effect of this treatment, and to decide when to stop opportunistic infection prophylaxis (2 - 4). For children, usually the percentage of CD4$^+$ T lymphocytes among total T lymphocytes and the CD4/CD8 ratio is required (5 - 7).

Flow cytometry (FCM) is the most widely accepted method for CD4$^+$ T lymphocytes enumeration. However, FCM instruments are expensive and the price of an FCM assay is relatively high. Although the dedicated CD4 flow cytometry systems (BD FACSCount, Guava Easy CD4 and Partec CyFlow) are less expensive, the costs of the instruments and the assays are not yet affordable for resource-constrained countries. Furthermore, the operation and maintenance of an FCM requires well-trained technicians and stable electricity. Alternative methods e.g. microbead separation methods (Coulter Cyto-Spheres and Dynal Dynabeads) are cheap, but they are labor-intensive and may have high operator-to-operator variations (8 - 10). SemiBio, a new system, applying microscope slides that have been treated to bind the CD4 cells from a blood sample, is under development (11, 12).

Recently, a few examples of miniaturized CD4 counting systems have been reported. Rodriguez et al. reported a microchip-based CD4 counting method for HIV monitoring in resource-poor settings. In this method, blood cells are stained with CD3 and CD4 fluorescent antibodies, captured on a membrane in a miniaturized flow cell, and then imaged through microscope optics (10). Thorslund et al. presented a concept of an inexpensive, polydimethylsiloxane (PDMS)-based point-of-care device for CD4 enumeration. The CD4$^+$ T lymphocytes from non-diluted whole blood were captured by
A CD3 Cell Immobilization Platform

surface modified PDMS. Detection was performed by a fluorescence microscope, where Hoechst33342 and CD3FITC were used to identify the captured CD4+ T lymphocytes (13). Cheng X et al. reported a microfluidic device for label-free CD4+ T lymphocyte counting. This device uses cell affinity chromatography operated under differential shear flow to specifically isolate CD4+ T lymphocytes directly from 10 μl of whole blood. CD4 counts are obtained by enumerating the cells captured on the chip under a microscope (14). Mishra et al. developed a micro-biosensor to capture the CD4+ T lymphocytes from lysed blood onto surface modified on-chip electrodes. The cells were recognized by increased impedance signals (15). Bachelder et al. proposed to utilize a quartz crystal microbalance for quantifying the CD4+ T lymphocyte counts. CD4 monoclonal antibodies (MAbs) were attached to the surface of a gold coated quartz crystal for use in a quartz crystal microbalance, which was able to quantify the amount of CD4+ T lymphocytes in PBS (16). In conclusion, the urgent need of affordable CD4 enumeration facilities has been stimulating the development of new CD4 enumeration technologies, many of which use surface bound antibodies to capture the CD4+ T lymphocytes. An important aspect of such systems is how to construct an interface that binds strongly and selectively to the target cells.

Reinhout et al. developed a technology to build up complex bio-structures consisting of a basic system, in which streptavidin (SAV) is bound to β-Cyclodextrin (βCD) self-assembled monolayers (SAMs) on a silicon oxide surface (17 - 20). The βCD is a well-known host for various small hydrophobic organic molecules in aqueous environments (21). The βCD is modified with seven heptathioether chains to obtain the ordered and densely packed self-assembled monolayers (SAMs) on a gold or silicon oxide surface. These SAMs have been extensively characterized (18, 22). The stepwise adsorption of the SAV to the βCD SAMs via a divalent linker allows hetero-functionalization of the SAV.

In this chapter, we present a novel technology of building up a CD3+ T lymphocyte immobilization platform for CD4 and CD8 enumeration and give the results of some preliminary tests of this system. The technology to buildup complex bio-structures is applied to buildup a cell immobilization platform for cell enumeration by attaching
specific MAbs to the βCD SAMs. The MAbs can be attached in different schemes. In this study, two kinds of CD3 MAbs were applied, clone CRIS-7 and clone B-B12. The CRIS-7 is on average mono-biotinylated, and can directly be attached to SAV. The B-B12 has to be assembled via a “universal” Fc-receptor, such as protein A (PA) or protein G (PG). These proteins should have biotin functionality and be able to attach to the SAV. The advantage of binding to Fc-receptors is that the Fab fragments of the MAbs will be pointing upwards. Therefore a Fab fragments layer is formed at the surface of the platform. The Fab fragments of the MAbs can now easily interact with the antigens on the cell surface and eventually capture the specific cells at the surface of the platform.

In this study, first, dot blot experiments were performed to find the best choice of the Fc receptor (PA or PG) and the CD3 MAb (biotin-CRIS-7 or B-B12) to build up the CD3 MAb layers. Secondly, SPR experiments were performed to monitor the stepwise build-up of the SAV layer and the biotin-CRIS-7 layer, or the SAV layer, biotin-PG layer and the B-B12 layer on the βCD SAMs. Thirdly, a preliminary cell experiment was performed to investigate the capability of the B-B12 layer for capturing the target cells (CD3+ T lymphocytes). The CD3+ T lymphocytes, the CD3+CD4+ T lymphocytes and the CD3+CD8+ T lymphocytes were enumerated and the relationship between the number of cells put at the surface with the number of cells captured by the surface was studied.

MATERIALS AND METHODS

Dot Blot Experiment

Two CD3 antibodies were used. Mono-biotinylation of CRIS-7 (IgG2a,κ; 0.526 mg/ml; Antibody Store, the Netherlands) was performed at Immunicon (USA), and the antibody was designated as Biotin-CRIS7. B-B12 (IgG1,κ; 3.385 mg/ml) was purchased from Antibody Store (The Netherlands). Using Phosphate Buffered Saline (PBS, pH7.2) as dilution buffer, antibody solutions of different concentrations (0.5 × 10^3 ng/μl, 0.5 × 10^4 ng/μl and 0.5 × 10 ng/μl) were prepared.

Stock solutions of the ligands (ImmunoPure® Recomb® Protein A-Horseradish Peroxidase Conjugated (Protein A-HRP) and ImmunoPure® Protein G-Horseradish Peroxidase Conjugated (Protein G-HRP), Pierce, USA) were prepared in the dilution
buffer at a concentration of 1mg/ml. Working dilutions were prepared by diluting the ligands stock solutions 5,000 fold using the blocking reagent (0.05% Tween-20 and 1% bovine serum albumin (BSA, Sigma, USA) in PBS).

Experiment: Circles (4 mm diameter) were drawn on a Protran nitrocellulose transfer membrane (Pore size: 0.45 μm, Whatman GmbH, Germany) using a pencil to indicate the regions where the protein samples would be blotted. 2 μl of the CD3 antibody solutions (Biotin-CRIS7 or B-B12) (in total 1 × 10³ ng, 1 × 10² ng and 1 × 10 ng) were slowly spotted onto the nitrocellulose membrane at the center of the circle using a pipette with a narrow-mouth tip. The membrane was dried in air. The non-specific sites of the membrane were blocked by soaking the membrane in the blocking reagent for 1 h at RT. Afterwards, the membrane was taken out of the blocking reagent and incubated in the ligands (Protein A–HRP and Protein G–HRP, respectively) working dilutions for 1h at RT with shaking. After incubation, the membrane was rinsed with wash buffer (0.05% Tween-20 (Surfact-Amps20, product No. 28320, Sigma, USA) and 0.1% BSA in PBS). Then the membrane was washed by suspending it in the wash buffer with shaking. The wash buffer was replaced every 5min and the washing steps were repeated at least 4 ~ 6 times. The membrane was incubated in substrate working solution (mixing equal parts of the Stable Peroxide Solution and the Luminol/Enhancer Solution (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce, USA)) for 5 min (0.1 ml of the substrate working solution per cm² of membrane). After that, the chemiluminescent images of the blots were recorded by Kodak Image Station 2000MM (Kodak, USA).

Buildup of the CD3 MAb Layers on βCD SAMs

βCD SAMs on gold-coated substrate preparation

The gold-coated substrates (BK7 glass/2-4 nm Ti/50 nm Au, SSens B.V., Hengelo, The Netherlands) were cleaned by dipping them in piranha (1:3 mixture of concentrated H₂SO₄ and 30% H₂O₂) for 5 seconds. After thoroughly rinsing with Millipore water, they were placed for 10 min in absolute ethanol to remove the oxide layer. Subsequently the substrates were placed in a freshly prepared 0.1 mM solution of βCD heptathioether for
16 h at 60 °C. The samples were subsequently rinsed 3 times with CHCl₃, ethanol and Millipore water (18). Afterwards, the substrate was immersed in a 1 mM aqueous solution of 1-Biotin-3-(3, 5-di(tetraethylene glycol) adamantyl ether) benzyl amide (divalent adamantyl biotin linker) for 15 min at RT (20). They were then ready to be used for buildup of the antibody layer. All solvents used in the layer preparation were of p.a. grade.

Buildup of the biotin-CRIS-7 or the B-B12 layer to the SAV layer

The stepwise buildup of the biotin-CRIS-7 or the B-B12 on the SAV layer on the βCD SAMs was monitored by Surface Plasmon Resonance (SPR) measurements (20) (Resonant Probes GmbH, Germany). The βCD monolayer on the gold-coated substrate was mounted in a Teflon flow cell (with a volume of 3.9 × 10⁻² ml), and a continuous flow of 0.5 ml/min was used. A Reglo Digital MS-4/8 Flow pump (Ismatec, Switzerland) with four channels was used to pump the solutions through the Teflon cell via Tygon R3607 tubings (Ismatec, Switzerland) with a diameter of 0.76 mm.

Experiments were started after the baseline had become stable. Stabilization of the baseline was reached by extensive rinsing the gold substrates with 10 mM βCD in PBS, followed by rinsing with PBS containing 1 mM βCD. Subsequently, all proteins were flowed through the flow cell. The SAV (10⁻⁷ M) (Sigma, The Netherlands) was prepared in PBS with 1 mM of βCD, while other proteins were prepared in PBS. For the buildup of the CRIS-7 layer (Fig 2A, scheme A), the SAV and the biotin-CRIS-7 (10⁻⁸ M or 10⁻⁷ M) were flowed through successively. And for the B-B12 layer (Fig 2B, scheme B), the SAV, the biotin-PG (10⁻⁷ M) (Sigma, The Netherlands) and the B-B12 (10⁻⁷ M or 10⁻⁶ M) were flowed through successively. For SPR experiments a gold substrate had to be used, while for cells imaged by a fluorescence microscope, a silicon oxide substrate was used.

CD3, CD4 and CD8 Enumeration Using a CD3 Cell Immobilizing Layer

CD3 MAAb (B-B12) layer buildup on silicon oxide substrate

The βCD SAM on glass slides (r = 12 mm, Menzel GmbH, Germany) were prepared as described in reference (17). Then the glass slides were immersed in a 1 mM aqueous
solution of 1-Biotin-3-(3,5-di(tetraethylene glycol) adamantyl ether) benzyl amide) (divalent adamantyl biotin linker) for 15 min at RT (20). Subsequently they were mounted in the flow setup. Proteins ($10^{-7}$ M of SAV in PBS with 1 mM of BCD, $10^{-7}$ M of biotin-PG in PBS, and $10^{-7}$ M of CD3 MAb B-B12 in PBS) were successively flowed through at a speed of 0.4 ml/min. In between the flowing of different proteins, PBS (containing 1 mM BCD) was flowed to avoid interactions of the proteins in the solution. This ready-to-be-used CD3 MAb layer was stored in PBS in a Petri-dish (R = 16.8 mm, Greiner GmbH, Germany). The B-B12 layer to be used in the cell experiment was in the center of the glass slide and the area was $19.6 \text{ mm}^2$ ($3.14 \times 2.5 \text{ mm} \times 2.5 \text{ mm} = 19.6 \text{ mm}^2$).

PBMC isolation

Peripheral blood (20 ml) was collected into two heparin (Venoject Lithium Heparin, Terumo, Belgium) tubes from a healthy donor. PBMC (Peripheral Blood Mononuclear Cells) were isolated using the Ficoll-Paque™ Plus (GE Healthcare, UK) density separation method.

One tube of the peripheral blood (approximately 10 ml) is diluted with 12 ml PBS supplemented with 0.38% trisodium citrate (Merck, Germany) and 0.5% bovine serum albumin (Sigma, USA) (PBS-TNC-BSA). 6 ~ 7 ml of the diluted blood is brought onto a layer of 3 ml Ficoll with a density of 1.077 g/cm$^3$. The tube was centrifuged at 2200 rpm (1000 g) for 20 minutes at RT. The layer on top of the Ficoll (the mononuclear cells) was collected and centrifuged with ~ 40ml PBS-TNC-BSA once at 1500 rpm for 7 minutes at 4 °C, and once at 1400 rpm for 6 minutes at 4 °C. The mononuclear cells were re-suspended in RPMI 1640 (Gibco, Invitrogen, USA) supplemented with 10% fetal calf serum (FCS, Invitrogen, USA) and 1% Antibiotic-Antimycotic solution (100x, Invitrogen, USA), and incubated for 1h at 37 °C in a T75 culture flask (Greiner GmbH, Germany). The lymphocytes suspension (Monocytes adhere on the wall of flask) was collected and centrifuged at 1400 rpm for 6 minutes at 4 °C. The supernatant was removed and the cell pellet was re-suspended in 2 ml of PBS and kept on ice.
Chapter 8

Concentration of the isolated lymphocytes

TruCount method (BD Bioscience, USA): 20 μl of TriTEST Reagent (CD3 clone: SK7, isotype: mouse IgG1-κ)-FITC/CD4 (clone: SK3, isotype: mouse IgG1-κ)-PE/CD45 (clone: 2D1, isotype: mouse IgG1-κ)-PerCP) was added into the TruCount™ absolute count tube. 50 μl of the lymphocytes suspension was added into the tube and mixed. After 15 min incubation at RT in dark, 450 μl of PBS was added and mixed. The samples were analyzed on the FACSCalibur using CELLQUEST™ software.

Capture of the CD3+ T lymphocytes on the B-B12 layer

Four lymphocytes suspensions were prepared by diluting the original cell suspension with PBS at different ratios. They are expected to contain 2016 ± 45 CD3+ T lymphocytes/μl, 1005 ± 32 CD3+ T lymphocytes/μl, 503 ± 22 CD3+ T lymphocytes/μl and 248 ± 16 CD3+ T lymphocytes/μl, respectively. 1 ml of each cell suspension was seeded into one Petri-dish (R = 16.8 mm, Greiner GmbH, Germany), in which one glass slide (r = 12 mm) with the B-B12 layer was laying on the bottom, as illustrated in Figure 1.

The lymphocytes suspension was incubated on the B-B12 layer in the Petri-dish for 30 min at RT. Afterwards, the cell suspension was removed and the glass slide was washed 3 times by gently adding and aspirating ~ 3 ml PBS into the Petri-dish. It was assumed that during the incubation, all cells that were initially present in the volume of liquid above the glass slide will sediment on the slide. The CD3+ T lymphocytes will attach to the B-B12 layer in the center area of the glass slide.

FIG. 1. A schematic drawing of cell suspension seeded into a Petri-dish, in which one glass slide with the B-B12 layer was laying on the bottom.
Cell enumeration by immuno-labeling and image recording

About 230 μl of the reagents cocktail, containing 0.5 μl of 1 mg/ml Hoechst33342 (Invitrogen, USA), 20 μl of CD3PE (clone:UCHT1, IgG1,κ, BD Pharmingen, USA), 20 μl of CD4FITC (clone: RPA-T4, IgG1,κ, BD Pharmingen, USA), 40 μl of CD8APC (clone: RPA-T8, IgG1,κ, BD Pharmingen, USA), and 150 μl of PBS, was added into each Petri-dish and incubated for 30 min at RT in dark. The glass slide was analyzed in a fluorescence microscope (Nikon ECLIPSE E400, Japan).

For each sample 3 areas in the center of the glass slide, where the CD3 MAb layer was located, were imaged. On each area, a Hoechst33342, a CD4FITC, a CD3PE and a CD8APC image were recorded, respectively.

RESULTS

Dot Blot Experiment

This experiment was performed to find the best choice of the Fc receptor (PA or PG) and the CD3 MAbs (biotin-CRIS-7 or B-B12) to buildup the CD3 MAbs layers.

FIG. 2. Chemiluminescent images of the dot-blots on nitrocellulose transfer membranes. A: PA binds to biotin-CRIS-7 and B-B12; B: PG binds to biotin-CRIS-7 and B-B12.

Figure 2A shows that for biotin-CRIS-7 (IgG2a,κ), $1 \times 10^2$ ng was detectable, while for B-B12 (IgG1,κ), $1 \times 10^3$ ng could be detected. This result indicates that PA binds strongly to the Fc fragment of biotin-CRIS-7 but weakly to that of B-B12. Figure 2B shows that for both biotin-CRIS-7 and B-B12, $1 \times 10^2$ ng could be detected. Since PG
binds strongly to both biotin-CRIS-7 and B-B12, it will be used later for the buildup of the CD3 layer. All these experimental results agree well with the reference (23).

It was also shown that the biotin of biotin-CRIS-7 does not hinder the interaction between CRIS-7 (Mouse IgG: 155 kDa) (24) and PA or PG. Apparently it is because biotin is very small (244 Da) when compared to IgG. It means that the buildup of the biotin-CRIS-7 layer can be carried out in the same scheme as that of the B-B12 layer.

**CD3 MAb Layer Buildup Schemes**

The sizes and shapes of the different proteins were taken into account when considering the assembly of the different proteins on top of each other. SAV is 58 kDa (2.5 nm × 3 nm × 5 nm), and the spacing between two biotin-binding pockets is about 2 nm (25). PA and PG are globular proteins (45 kDa and 22 kDa, respectively) (26) with a diameter of approximately 6 nm and 3 nm respectively. This means that biotin-PG and biotin-PA (Fc receptors) can bind to SAV at a ratio of 1:1 or less. MAbs (IgG) are large molecules (~150 kDa) with a “Y” shape. Therefore the binding ratio of the MAb to the PA or the PG molecules is also at most 1:1. Eventually the binding ratio of the MAb to SAV is at most 1:1, no matter whether it is bound directly by the biotinylated MAb or indirectly by the biotinylated PA or PG.

**FIG. 3.** The attachment of biotin-CRIS-7 and B-B12 in different schemes to the βCD SAMs. A: Biotin-CRIS-7 is bound through the biotin functionality to the SAV layer on
the βCD SAMs; B: B-B12 is bound to the Fc receptor PG, which is biotinylated and bound to the SAV layer on the βCD SAMs.

Figure 3 illustrates the schemes of the biotin-CRIS-7 (A) and the B-B12 (B) layers buildup. The biotin-CRIS-7 is bound through the biotin functionality to the SAV layer on the βCD SAMs. The B-B12 is bound to the Fc receptor PG, which is biotinylated and bound to the SAV layer on the βCD SAMs.

Monitoring of the CD3 MAb Layer Buildup by SPR Experiment

SPR experiments were performed to monitor the stepwise buildup of the biotin-CRIS-7 layer or the B-B12 layer on the SAV layer on top of the βCD SAMs layer.

**FIG. 4.** SPR sensograms displaying the adsorption of SAV to βCD SAMs, followed by A: the adsorption of $1 \times 10^{-8}$ M of biotin-CRIS-7 to the SAV; B: the adsorption of $1 \times 10^{-7}$ M of biotin-CRIS-7 to the SAV; C: the adsorption of biotin-PG with subsequent adsorption.
The SPR sensorgrams (Figure 4) display the different successful buildup processes. The biotin-CRIS-7 formed a dense layer on top of the SAV layer when its concentration was $1 \times 10^{-7}$ M, while $1 \times 10^{-6}$ M did not saturate the SAV binding. The B-B12 also formed a layer on top of the biotin-PG at the SAV layer. Similarly, the B-B12 layer was much denser at a higher concentration ($1 \times 10^{-6}$ M).

The advantage of the scheme B in Figure 3 is that by using the Fc-receptor biotin-PG, the Fab fragments of the B-B12 MAbs are pointing upwards. Thereby the Fab fragments of the MAbs can easily interact with antigens on the cell surface and eventually easily capture the CD3 cells on the surface of the platform. While in the scheme A, the Fab fragments of the biotin-CRIS-7 MAbs are probably not exactly pointing upwards, since the biotin molecule may be anywhere on CRIS-7 MAb. There are more than 100 free amino groups to which biotin can bind. Therefore the B-B12 will be used as CD3 MAb for future cell experiment.

**CD3, CD4, and CD8 Enumeration Using a CD3 Cell Immobilizing Layer**

In the test experiments, we used $1 \times 10^{-7}$ M B-B12 to buildup the CD3 cell immobilization layer, since a separate cell experiment (not presented here) showed that there was no difference in capturing CD3 cells between the B-B12 layer made by $1 \times 10^{-6}$ M and by $1 \times 10^{-7}$ M of B-B12. The reason is probably the steric hindrance of antigen access to the antibody binding sites (Fab fragments) caused by the decrease in antigen binding capacity with increasing surface density of antibody (23, 27).

First, the specificity of the capturing of cells by the B-B12 layer was determined by labeling the cells at the surface with Hoechst 33342 and CD3PE. The number of Hoechst33342 stained cells and the number of CD3PE stained cells were compared for all samples (see below). Hoechst33342 is a DNA dye, which stained all the cells at the
A CD3 Cell Immobilization Platform

B-B12 layer. The specificity appeared to be more than 80%, since it was found that 80 – 90% of the Hoechst stained cells were also CD3PE fluorescent.

Next, the suitability of the CD3 MAb layer for cell enumeration was evaluated. CD3⁺ T lymphocytes, CD3⁺CD4⁺ T lymphocytes and CD3⁺CD8⁺ T lymphocytes were enumerated and the relationship between the number of cells put in and the number of cells captured was determined.

![Fluorescent images of captured cells](image)

**FIG. 5.** Images of one area of the sample with the highest cell concentration of the lymphocytes suspension. A: Color merged image of the CD3PE (red) and the CD4FITC (green) images. B: Color merged image of the CD3PE (red) and the CD8APC (cyan) images. C: Color merged image of the CD4FITC (green) and the CD8APC (magenta) images. (color figures on Page 213)

Figure 5 shows the fluorescent images of the captured CD3⁺ T lymphocytes on the B-B12 layer platforms seeded with the highest concentration of the lymphocytes suspension. The immobilization of different cell types is shown. The CD3⁺CD4⁺ T lymphocytes are recognized by both the CD4FITC and the CD3PE fluorescence, and the CD3⁺CD8⁺ T lymphocytes with both the CD8APC and the CD3PE fluorescence. Subsequently, the images were analyzed to obtain cell counts.

The concentration of the lymphocytes suspension determined by the flow cytometry TruCount method was 9481 CD45 leukocytes/µl, which contained 8508 lymphocytes/µl (89.74% of CD45 leukocytes), 6204 CD3⁺ T lymphocytes/µl (65.44% of CD45 leukocytes), 3530 CD3⁺CD4⁺ T lymphocytes/µl (37.24% of CD45 leukocytes), and 2674 CD3⁺CD8⁺ T lymphocytes/µl (28.20% of CD45 leukocytes). Four lymphocytes suspensions were made by diluting the original cell suspension with PBS in different
ratios. They are expected to be $2016 \pm 45$ CD3$^+$ T lymphocytes/µl, $1005 \pm 32$ CD3$^+$ T lymphocytes/µl, $503 \pm 22$ CD3$^+$ T lymphocytes/µl and $248 \pm 16$ CD3$^+$ T lymphocytes/µl, respectively.

Assuming that all the CD3$^+$ T lymphocytes put on the B-B12 layer adsorb to the surface, the expected number of cells/image can be calculated from the known dimensions of slide and Petri-dish. Dimensions are: Petri-dish: $R = 16.8$ mm; Glass slide: $h = 0.17$ mm, $r = 12$ mm; and Image size: $0.68$ mm $\times 0.84$ mm. The height of cell suspension in Petri-dish ($H$) is $1.22$ mm by calculation. So the height of cell suspension on top of glass slide is $1.05$ mm. The expected number of cells per image is equal to the image size $\times 1.05$ mm $\times$ cell suspension concentration ($0.59$ (µl/image) $\times$ cell concentration (#/µl)).

Figure 6A-C shows the number of captured cells counted by a fluorescence microscope as a function of the number of expected cells calculated as described above. Error bars are the Square Root (SQRT) of the expected cell number and the Standard Deviation (SD) of the captured cell numbers of 3 areas. It is shown that the captured number increased with the expected number. The trend of the four points is close to that of the ideal condition as shown by the line (the captured number is equal to the expected number). The relative deviations between the captured number and the expected number are larger at the lower cell numbers, probably in part due to the inaccurate geometry which may lead to systematic errors. The reason of the deviation may also be the difference between the assumption and the practice. The expected cell number is calculated by assuming that all the CD3$^+$ T lymphocytes are captured on the B-B12 layer. However, in practice, this preliminary cell experiment may not have achieved 100% binding.
FIG. 6. The number of captured cells counted by a fluorescence microscope as a function of the number of expected cells calculated by FCM measurement. A: CD3\(^+\) T lymphocytes; B: CD3\(^+\)CD4\(^+\) T lymphocytes, and C: CD3\(^+\)CD8\(^+\) T lymphocytes. To guide the eye, a line with a slope of 1 is shown, in which both values are same. The error bars are the SQRT of the expected cell number and the SD of the captured cell numbers of 3 areas.

DISCUSSION

Immobilization of MAbs on solid surfaces is the basic step of many immunoassay techniques. However, a very common technical problem is the low antigen binding capacity when antibody is immobilized onto a solid substrate. Upon adsorption on solid surfaces, a MAb molecule can partly or even completely lose its ability to bind antigen. This adsorption induced reduction in the effective immunological activity can be caused by changes in the protein structure and/or by an unfavorable orientation of the antigen binding sites on the solid surface reducing antigen accessibility because of steric hindrance (27 - 31). The CD3 MAbs immobilizing method presented in this chapter may solve this common problem. The advantage of building up a CD3 MAbs layer on CD SAMs using the presented two schemes is that the orientation of the antigen binding region on the Fab fragments of IgG is directed towards the solution. Especially in the scheme B using the Fc-receptor protein G, the Fab fragments of MAbs should be pointing upwards. Therefore a Fab fragments layer should be formed on the surface of the platform that can easily interact with the antigens on the cell surface and eventually capture the specific cells at the surface of the platform.
The results of these preliminary cell experiments indicate that the B-B12 layers investigated are indeed suited, but further experiments are needed. We expect that a novel CD4 and CD8 enumeration method can be developed based on the cell capturing MAb layer attached on the βCD SAMs (17 - 20) in combination with our single-platform image cytometer (SP ICM) (32 - 34). The CD3⁺ T lymphocytes from PBMC can be captured by the CD3 MAb layer buildup and the CD4 and the CD8 immuno-fluorescent labeling can be imaged by SP ICM to enumerate the CD3⁺CD4⁺ T lymphocytes and the CD3⁺CD8⁺ T lymphocytes. To develop a complete device for CD3, CD4 and CD8 enumeration, a microfluidics chamber should be developed, in which the CD3 MAbS layer platform is mounted. To improve the efficiency and specificity of capturing the specific cells on the MAb layers, a miniaturized flow system should be implemented, which can control the introduction of whole blood, followed by the introduction of washing buffer to remove the non-specific cells, and of the staining reagents at an optimal flow rate. Maybe the concentration of the CD3 MAbS, used to buildup the cell capturing MAbS layer platform, should also be optimized to have better binding capacity, and better blocking for non-specific binding.

This approach would especially be attractive if the layer system could be used to adsorb the target cells from whole blood, as a part of a dipstick sensor, and be read out by our ICM.

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9.1 Outlook

The goal of this project is to develop an affordable, reliable and easy-to-use system for HIV staging in point-of-care settings of resource-constrained countries. We have developed four fully functioning StarCount systems, SC 1.0, SC 2.0, SC 2.1 and SC 3.0, for the enumeration of well-defined populations of white blood cells, most importantly, CD4+ T
lymphocytes (1-3). The whole system consists of an instrument and a sample preparation procedure, and has been designed to be simple and easy-to-use, even for less-trained operators. Sample preparation utilizes a no-lyse, no-wash procedure and requires only 3 pipetting steps. The instrument is fully automatic and computer controlled, suitable to be used by less-trained personnel. It is portable, robust, and operates stand-alone on a rechargeable battery, even on a car battery. It can be operated immediately after arrival at the testing site, and does not require alignment or calibration. For the SC 2.1, suited for CD4+ T and CD8+ T lymphocytes enumeration, the instrument costs ~ US$ 3,000 (single-unit component price) and the cost per assay is ~US$ 3. This price is more affordable than the dedicated CD4 (and CD8) FCM methods. The results of the patients are available in about 45 min after blood draw. Accordingly, the clinician can make a treatment decision at the time a patient visits. One well-trained operator can carry out at least 30 tests for both CD4 and CD8 enumeration with one instrument and several MagNest® in 8 hrs. All these aspects are especially advantageous for point-of-care usage in resource-poor countries. SC 2.1 has been evaluated on ~500 HIV+ patients (460 adults and 47 children) in Thailand and the results showed that SC 2.1 is comparable to the gold standard SP FACSCount and DP FACSCan methods for blood samples with CD4 counts ranging from 1 to ~ 2000/µl, most importantly in the low CD4 count range (<200/µl). The present studies were performed by the persons that developed the system. To gain international acceptance, a multi-center study need to be conducted in which the systems are operated by the personnel at the sites and preferably in resource-limited settings. At present studies are being planned in Rwanda, Venezuela and Burma. Successful evaluation at these sites will warrant a multi-center study.

Although both SC 2.0 and SC 2.1 have been validated to be very good alternative CD4 enumeration methods to replace the expensive SP and DP FCM methods in point-of-care settings of resource-constrained countries, there are still some aspects that could be improved.

First, for pediatric HIV staging, the CD4/CD8 ratio is a useful parameter for early diagnosis and identification of infants infected with HIV (4), however for the initiation of ART therapy the % of CD4+ T lymphocytes of total lymphocytes is more informative (5). Our current systems do not measure the total lymphocytes count, therefore various approaches that may provide a total lymphocytes count will need to be evaluated. For example, the SC 3.0 system may be adapted to obtain a % of CD4+ T lymphocytes of total
lymphocytes. CD45-magnetic selection in combination with cell stimulation, and Rhodamine123 and Hoechst33342 staining can be used. In this case the total lymphocytes count can be obtained by subtraction of cell counts from two images: the Hoechst33342 image showing all leukocytes, and the Rhodamine123 image showing all monocytes and granulocytes.

Secondly, a quality control is needed to ensure the satisfactory performance of the SP ICM systems as well as the assay. Proper and cost effective controls will need to be developed to assure that the systems and the assay are indeed working according to predefined specifications.

Thirdly, as a point-of-care system, the current SC 2.0 and SC 2.1 work well on fresh blood specimens (less than 12 hours after blood draw). For blood specimens of healthy donors, the CD4⁺ T lymphocytes could reliably be enumerated within a time period of 72 hrs after blood draw; however, for some HIV⁺ patients the SC 2.0 system (CD4FF/CD3PE) gives CD4 over-counts when the blood specimens were aged over 24 hrs (2). This over-count is most likely caused by the fact that for aged blood samples of some HIV⁺ patients, a part of the CD3⁺CD8⁻ T lymphocytes are non-specifically (magnetically) selected, and counted as CD4⁺ T lymphocytes since they are labeled with CD3PE. Such non-specific selection probably arises due to the abnormalities of the immune system in HIV⁺ patients (6). The non-specific adhesion of FF particles plays an important role in this CD4 over-count phenomenon. To overcome this problem in aged blood specimens from HIV⁺ patients, we have tested commercially available blood cell preservative products, i.e. CellSave™ Cell Preservative Tube (Immunicon Inc., USA) (7), Cyto-Chex (Streck Laboratories, USA) (8 - 13) and TransFix (Cytomark, UK) (14 - 16). The CellSave™ Cell Preservative Tube was used directly for blood draw and storage. The Cyto-Chex and the TransFix were used by adding it into the EDTA anti-coagulated blood to help store the blood cells. The TransFix is the only product showing promising results on preventing the CD4 over-count in aged blood specimens from HIV⁺ patients. Nevertheless, the core of this over-count problem is the changes that are introduced when blood cells are stored and the interaction of these changed cells with the FF magnetic particles. A method using no FF might give an improvement.

Fourthly, the cause of the CD8 under-count in the SC 2.1 system was shown to be mainly due to the low sensitivity of the SC 2.1 for the detection of the CD8⁺dim T lymphocytes when using the current sample chamber that has a depth of 4 mm. It has been noted that the
unbound magnetic particles (FF) tend to aggregate at the upper-surface of the chamber and are visible as lines that hamper the cell recognition by the image analysis algorithm. This is another reason to avoid the use of magnetic particles in a next version of the SP ICM system. The loss in CD8 count may partly be due to the fact that some CD8 cells have not reached the surface within the 20 min magnetic separation time, because of insufficient magnetically labeling. Although we do not yet have direct proof for this hypothesis, avoiding magnetic selection may at least prevent this from happening. Therefore, there is a strong need to go for a non-magnetic particles approach.

Fifthly, the chamber used in the current SC systems is designed for rare cell magnetic enrichment application and has a depth of 4 mm. This large depth diminishes the quality of the immuno-fluorescence image, since the unbound fluorophores in the depth introduce a high background in the image. More than half of the CCD dynamic range or CCD full well capacity was occupied by the background; this probably conceal the very dim CD8^{\text{dim}} T lymphocytes. A preliminary test, using a thin chamber (ViaSure slides, Immunicon Inc.) with 140 \mu m well depth and PerCP beads with different intensities, was performed. The results indicate that the whole range of CD8^{\text{dim}} T lymphocytes can be detected when CD8^{\text{bright}} T lymphocytes are also present. Therefore, a thin chamber should be developed for use in a new design of the SP ICM system.

An ideal design would be one that integrates a microfluidics chip with a detector, combining the whole blood collection, the mixing with reagents, and the imaging in one device. Whole blood would be collected by pressing micro-needles against the patient’s skin. The blood may then be transferred through a mixing channel where the lyophilized reagents have been introduced. Afterwards, the blood sample reaches an image well where the stained target cells are imaged.

As a first step towards a microfluidics integrated device, the development of a new cell-imaging chamber has been started. A new cell imaging chamber with a depth of 200 \mu m was designed and fabricated in our group by using poly(dimethylsiloxane) (PDMS). This cell-imaging chamber was prepared by pouring a pre-polymer mixture into a mold and curing the pre-polymer to create a PDMS structure. A cover-glass is put on top to cover the chamber well before using. Another new glass slide, having 6 wells with 140 \mu m well depth, was developed by Immunicon. These two sets of new cell-imaging chambers and slides are thin enough to image all cells that have sunk to the bottom. The magnetic separation step can
therefore be eliminated. There are some other possible merits of using these new thin chambers and slides. First, the reduced depth reduces the amount of free fluorophores in the field of view; therefore the image quality could be improved. The whole range of the CD8\(^{dim}\) T lymphocytes may then probably be detected and counted. The CD8 enumeration by the SP ICM may achieve the same efficiency as the FCM does. Secondly, a much smaller volume of reagents is needed, leading to a reduced test cost. Thirdly, replacement of the expensive 4 mm Immunicon sample chamber (~ US$ 1.8) by the cheap new chamber and glass slide (~US$ 0.8 per slide for 6 tests) cuts about half of the current CD4 enumeration test cost. There will be no need for a MagNest\(^\circledR\) (US$ 150/piece), which will greatly reduce the instrument cost.

For application of these thin chambers and slides on CD4 and CD8 enumeration, CD4\(^+\) T lymphocytes are defined by both CD3\(^+\) and CD4\(^+\) staining, either CD3PE/CD4PerCP or CD4PE/CD3PerCP and CD8\(^+\) T lymphocytes are defined by both CD3\(^+\) and CD8\(^+\) staining, CD8PE/CD3PerCP. In this way, all CD8\(^+\) cells have to be checked by the positive CD3PerCP staining to exclude the CD3\(^-\)CD8\(^+\) NK cells. The other way of staining, i.e. CD3PE/CD8PerCP, is not a proper one as the cell image with different CD8PerCP intensities will be interfered by the cross-talked CD3PE image.

The Immunicon 6-well glass slide is ready to be used with the EasyCount instrument for CD4 and CD8 enumeration. The EasyCount instrument applies the same principle as the SC 2.1 instrument for both CD4 and CD8 enumeration; however, it is much more expensive than the SC 2.1, since it is equipped with a computer controlled filter changer and a computer controlled sample chamber moving stage for scanning the preset position on the surface of the chamber. This whole system will soon be tested together with our SC 2.1 on HIV\(^+\) patients in Rwanda.

A new SP ICM system, NewCount, is currently under development. This system combines the merits of the thin chambers and slides and those of the lower magnification optic system, SC 2.5 (as shown in Figure 1). Currently three images are taken in SC 2.0 and SC 2.1 system by manually moving the chamber position in the MagNest\(^\circledR\). A low magnification optic system will be implemented in the NewCount system to allow enough cells, needed for sufficient statistics, in one image. Furthermore, alternative illumination systems (optical fiber, laser diode, alternative LEDs) will be tested to have a better homogeneity and brightness than the current one. As a battery supported stand-alone system, decreased power
consumption in the NewCount is another concern. As a portable instrument, a miniaturized dimension is important. Therefore an alternative camera, a new SBC, a smaller display and a PDA will be tested. Further reduction in the instrument cost is one of the main goals of the NewCount development. We expect to build a NewCount instrument with a dimension of 15 cm × 15 cm × 20 cm, a power consumption of ~30% of that of the SC 2.1, and a single piece components cost of ~US$ 1,500.

As a point-of-care system for application in resource-poor countries, the stabilization and packaging of the reagents for stability under tropical conditions is a necessary concern. The lyophilization of MAb-fluorophores has to be realized.

The final goal: an instrument cost of US$ 1,000, test cost US$ 1.0, no moving parts, temperature and humidity resistance, is within reach.

9.2 Summary of the Thesis
HIV infection has become a major global pandemic health emergency since the official date of the first recognition of the AIDS epidemic in Los Angeles in 1981 (17). This epidemic has formed a serious, and in many countries, especially in resource-poor countries, devastating crisis. Recent years, the generic antiretroviral treatment (ART) has become more and more available to resource-poor countries (18). However, the patients are only allowed to have the ART when their CD4+ T lymphocytes count in the peripheral blood is known. The CD4+ T lymphocytes count is the most important clinic parameter to determine the disease stage and progression, to assist in making decisions regarding when to start or change ART, and to assess the effect of treatment (19 - 21). Besides, the CD4 percentage in the total lymphocytes or CD4/CD8 ratio is also required for pediatric HIV monitoring (22 - 26).

Flow cytometry is the gold standard method for the enumeration of CD4+ T lymphocytes and CD8+ T lymphocytes. However, it is still too expensive for and inaccessible to many resource-poor countries (especially in the Sub-Saharan African countries). Although the dedicated flow cytometers for CD4 and/or CD8 enumeration (BD FACSCount, Guava PCA, Partec CyFlow, PointCare FlowCare) have reduced the costs, development of more affordable, simpler, more easy-to-use and point-of-care CD4 and CD8 enumeration system for resource-poor countries is still urgently needed (27 - 29).
This thesis presents the development, testing and evaluation of a series of Single Platform Image Cytometer systems (SP ICM), named StarCount (SC), for the selection, recognition and enumeration of well-defined populations of white blood cells, most importantly the CD4⁺ T lymphocytes. The technology is based on magnetic selection and fluorescent labeling of target cells, which are then imaged on an automated fluorescence ICM. Several different instruments have been developed and a number of immunochemical procedures have been tested in this thesis.

Chapter 2 gives a detailed description of the immunochemical method and instrumentation of our system. In the Immunochemical Methods part, the first StarCount system, StarCount 1.0 (previously called EasyCount), was taken as an example. The sample preparation was designed to be simple and easy, even for less-trained personnel. It uses a no-lyse, no-wash procedure and requires minimal pipetting steps. The amount of reagents consumed is kept small so that the test cost is low. The reagent incubation time, the DNA fluorescent labeling, the sample chamber, the dilution factor, the type of immuno-magnetic particles, and the magnetic separation time were discussed. The experimental parameters for different StarCount systems were summarized at the end of this part. In the instrumentation part, StarCount 2.0, the first prototype that gave reliable CD4 counts was used as an example to address the functional design, the factors determining the sensitivity, the components (illumination unit, image optics, CCD camera, single board computer and touch-screen, power supply and housing), the image analysis algorithm, and the software shell (3). The design criteria are that the instrument should have a high sensitivity, good selectivity and reliable performance. It also has to be affordable, easy-to-use even for less-trained personnel, portable, robust, and should be able to run on a rechargeable battery. An overview of the instrument settings for different StarCount systems was given at the end of the chapter.

In chapter 3, the first system, StarCount 1.0 is discussed (1). The instrument was designed to image and enumerate the immuno-magnetically selected cells stained with a nucleic acid dye, acridine orange. We evaluated the performance of this system in enumerating CD45⁺ leukocytes, CD3⁺ T lymphocytes, and CD19⁺ B lymphocytes in human whole blood. These cell populations can be well defined by one kind of antibody. By comparing the cell counts obtained from SC1.0 to those from single-platform (SP) (TruCount) and dual-platform (DP) flow cytometry (FCM) methods, the accuracy of our system was assessed. Excellent precision and linearity were shown. We also addressed that in case the applied
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immuno-magnetic label is not specific for only one type of cell subpopulation, as for CD4+ cells, both CD4+ T lymphocytes and CD4dim monocytes are selected. Additional immuno-labeling should be applied to accurately enumerate CD4+ T lymphocytes for HIV staging.

The realization of SC2.0, the first accurate CD4+ T lymphocytes enumeration system, is discussed in chapter 4 (2, 3). This system identifies CD4+ T lymphocytes as the CD3+CD4+ population. One antibody is used for immuno-magnetic selection, and the other for immuno-fluorescent labeling. In this way CD4dim monocytes are excluded. The fact that the selected cells are positive for two antibodies brings in the option of two different labeling schemes: CD4-ferrofluid (CD4FF)/CD3-Phycocerythrin (CD3PE) and CD3FF/CD4PE. Both schemes gave good results, exchangeable between each other, and more importantly, with the two SP FCM methods (tetraCXP and TruCount methods) that are frequently used as gold standard. The excellent precision, the accuracy and the linearity of the SP ICM in the clinically important range of CD4+ T lymphocytes counts (0 – 500/µl) for monitoring HIV induced immunodeficiency were achieved.

The further development of SC 2.0 leads to SC 2.1, a system that is capable of both CD4 and CD8 enumeration and CD4/CD8 ratio determination. This system is dedicated for pediatric HIV monitoring (Chapter 5). Only one-step immuno-labeling of whole blood with CD3-magnetic nanoparticles, CD4-Phycocerythrin (PE) and CD8-Peridinin-Chlorophyll-III-Protein Complex (PerCP) is required in the sample preparation. The sensitivity of the SP ICM for detecting CD8bright T lymphocytes and CD8dim T lymphocytes simultaneously was investigated using conjugated PerCP-magnetic beads with different intensities. The influence of PE fluorescence cross-talk in the PerCP image on CD8 enumeration was also discussed. A certain level of CD8 under-count in the SC2.1 system was expected.

We compared CD4, CD8 count and CD4/CD8 ratio by the SP ICM with those by a SP FCM tetraCXP method on blood samples from 50 HIV− adults, 50 HIV+ adults and 45 HIV− children. Good correlations were obtained for all the measurements. CD4 counts by the SP ICM were comparable to those by the SP FCM, whereas CD8 counts by the SP ICM were about 10% lower than those by the SP FCM. The CD8 under-count was partly because of a small percentage of CD8dim T lymphocytes (~ 10%) that were either not detected by the SP
ICM or not counted due to the interference of the cross-talked PE image of CD4+ T lymphocytes.

In order to have a fully automated system for less trained operators, two new automatic image analysis algorithms, the dynamic threshold method and the maximum entropy method were developed and tested in this study. Both performed comparably with the trained operator manual threshold method. The dynamic threshold method showed a slightly larger deviation. Most importantly, it is time-consuming and certainly not suitable for application in a busy routine lab. The maximum entropy threshold method was found to be very promising and has been tested extensively in a large clinic trial in Bangkok (see Chapter 7).

In chapter 6, we present the development of the immunocytochemical methodology of a novel and affordable CD4 enumeration method. This method combines immuno-magnetic selection of CD4 positive cells using only CD4 antibody and a cell activation strategy to distinguish the co-selected monocytes and granulocytes from CD4+ T lymphocytes. Whole blood samples were treated with the DNA dye Hoechst33342, the oxidative burst stimulator Phorbol 12-myristate 13-acetate, and Dihydrorhodamine123. By CD4 immuno-magnetic labeling, CD4+ T lymphocytes, CD4dim monocytes and some non-specifically bound granulocytes are magnetically attracted to an analysis surface of a chamber with a known volume. Two fluorescent images of the same area are recorded and analyzed; a Hoechst33342 image (DNA image) shows all magnetically attracted leukocytes at the surface, and a Rhodamine123 image shows activated monocytes and activated granulocytes (mainly neutrophils). By subtracting the cell counts obtained from these 2 images, the absolute CD4+ T lymphocytes count can be obtained. The principle of this system was verified on blood samples of 29 healthy donors using a fluorescence microscope. Furthermore, no difference in the oxidative burst activation kinetics between 42 HIV+ patients and 18 healthy donors was found using FCM. A dedicated simple SP ICM instrument (SC3.0) was accordingly built, tested and evaluated by comparing the CD4+ T lymphocytes counts of blood samples of 42 HIV+ patients with the counts obtained by the SP FCM. A reasonably good correlation was achieved. Bland-Altman plots showed interchange-ability between the SP ICM method and the SP FCM methods.

Chapter 7 presents the results of a field test of the SC2.1 system on HIV infected patients carried out in Siriraj Hospital, Bangkok, Thailand. CD4, CD8 counts and CD4/CD8 ratios in whole blood samples of 460 HIV+ adults and 47 HIV+ children were obtained by the SC2.1.
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The maximum entropy threshold method was applied. The results were compared with those obtained by a SP FCM FACSCount method and by a dual platform (DP) FCM FACSCan method, respectively. CD4 counts by the SP ICM were comparable ($R \geq 0.97$) with those by the FACSCount and the FACSCan with a bias of 9.1% and 11.2%, respectively. In the very low CD4 count range ($\leq 50\mu l$), there were some over-count outliers by the FACSCan and the SP ICM, if the FACSCount was considered as the gold standard. This over-count will unlikely influence the making of clinical decision nor affect the monitoring of HIV status, as long as the same method is used all the time for patients. For CD8 enumeration, the SP ICM showed a consistent under-count (~15%) as compared to the FACSCount, almost same as we found previously in chapter 5. From this big clinical trial, we concluded that a fully automated SP ICM can be made by implementing the maximum entropy method in the macro. The SP ICM will then be a very good alternative method for the expensive SP and DP FCM methods in point-of-care settings in resource-constrained countries.

In chapter 8, we present the principle of a novel technology of building up a CD3$^+$ T lymphocytes immobilization platform for CD4 and CD8 enumeration. Complex bio-structures, in which streptavidin (SAV) is bound to $\beta$-Cyclodextrin ($\beta$CD) self-assembled monolayers (SAMs) on silicon oxide surface, were formed. CD3 monoclonal antibodies (MAbs) were attached to the $\beta$CD SAMs via the SAV to form a CD3 cell immobilization platform. Dot blot experiments indicated that biotin-protein G (biotin-PG) is suitable as an Fc receptor for the CD3 Monoclonal Antibody (MAb) (B-B12 or biotin-CRIS-7) to build up the CD3 MAb layers. Surface Plasmon Resonance (SPR) sensograms showed a successful stepwise build-up of a biotin-CRIS-7 layer and B-B12 layer on the $\beta$CD SAMs in two different schemes. A preliminary cell experiment was performed to investigate the ability of the B-B12 layer to capture the CD3$^+$ T lymphocytes. The CD3$^+$ T lymphocytes, the CD3$^+$CD4$^+$ T lymphocytes and the CD3$^+$CD8$^+$ T lymphocytes were enumerated and the relationship between the number of cells introduced and the number of cells counted (captured cells) was studied. The results of these preliminary cell experiments indicate that the B-B12 layers investigated are indeed suited for CD4 and CD8 enumeration. To draw more definite conclusion, further experiments are needed. We expect that a novel CD4 and CD8 enumeration method can be developed based on the cell capturing MAb layer attached on the $\beta$CD SAMs (30-33) in combination with our SP ICM (1 - 3).
Outlook and Summary

In conclusion, the SP ICM we developed is a good CD4 and CD8 enumeration system for HIV staging in point-of-care settings of resource-constrained countries.

REFERENCES


Outlook and Summary


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SAMENVATTING

HIV infectie is een groeiend wereldwijd probleem sinds de officiële erkenning van de AIDS epidemie in Los Angeles in 1981 (17). Deze epidemie heeft in veel arme landen tot een verwoestende crisis geleid. In de afgelopen jaren zijn antiretrovirale behandelmethoden (ART) steeds beter beschikbaar geworden in arme landen (18). Patiënten kunnen echter alleen behandeld worden als de concentratie van CD4+ T lymfocyten in hun bloed bekend is. De concentratie van CD4+ T lymfocyten is de belangrijkste klinische parameter bij het bepalen van de status en vooruitgang van de ziekte, om te bepalen wanneer antiretrovirale behandeling gestart of aangepast moet worden en om het effect van de behandeling te monitoren (19 - 21). Ook is het percentage CD4 cellen van het totaal aantal lymfocyten of de CD4/CD8 verhouding nodig voor het volgen van HIV infectie bij kinderen (22 - 26).

Flow cytometrie is de gouden standaard voor het bepalen van de concentratie CD4+ T lymfocyten en CD8+ T lymfocyten. Deze techniek is echter nog steeds te duur en niet toegankelijk voor arme landen (vooral landen in Afrika ten zuiden van de Sahara). Toegewijde flow cytometers voor het bepalen van de CD4 en/of CD8 concentratie (BD FACSCount, Guava PCA, Partec CyFlow, PointCare FlowCare) hebben de kosten al aardig weten te drukken. Het ontwikkelen van een betaalbaar, eenvoudiger en gemakkelijker te gebruiken systeem voor het ter plekken bepalen van CD4 en CD8 concentraties in arme landen is dringend nodig (27 - 29).

Dit proefschrift toont de ontwikkeling, het testen en de evaluatie van een serie van Single Platform Image Cytometer systemen (SP ICM), genaamd StarCount (SC), voor het selecteren, herkennen en het bepalen van de concentratie van goed gedefinieerde populaaties witte bloedcellen, vooral CD4+ T lymfocyten. De technologie die hierbij wordt gebruikt is gebaseerd op magnetische selectie en fluorescente labeling van specifieke cel typen, die vervolgens worden afgebeeld met behulp van een geautomatiseerde fluorescentie microscoop. Meerdere instrumenten zijn ontwikkeld en verscheidene immuno-chemische procedures zijn getest in dit proefschrift.

Hoofdstuk 2 geeft een gedetailleerde beschrijving van de immuno-chemische methode en de instrumentatie van ons systeem. In het gedeelte dat gaat over de immuno-chemische
methode word het eerste StarCount systeem, StarCount 1.0 (voorheen EasyCount), als voorbeeld gebruikt. Het bereiden van monsters is ontworpen om makkelijk en snel te zijn, ook voor minder getraind personeel. Hierbij word een methode gebruikt waarbij niet gelyseerd of gewassen hoeft te worden en het aantal pipetteer stappen word tot een minimum beperkt. De gebruikte hoeveelheid reagentia is geminimaliseerd om de kosten per test te drukken. De incubatietijd van de reagentia, het fluorescent labelen van het DNA, de sample-kamer, de verdunningsfactor, het type immuno-magnetische deeltjes en de tijd voor magnetische scheiding worden besproken. De experimentele parameters voor verschillende StarCount systemen zijn samengevat aan het eind van dit gedeelte. In het gedeelte over de instrumentatie is de StarCount 2.0, het eerste prototype dat betrouwbare CD4 concentraties opleverde, gebruikt als voorbeeld om het functionele ontwerp, de factoren die gevoeligheid bepalen, de componenten (belichtingseenheid, afbeeldende optiek, CCD camera, kleine computer en touch screen, voeding en behuizing), het analyse algorithme en de bedienings software te bespreken (3). De ontwerpcriteria zijn een hoge gevoeligheid, goede selectiviteit en betrouwbaarheid. Het systeem moet ook relatief goedkoop zijn, gemakkelijk te gebruiken zelfs voor minder getraind personeel, draagbaar en robuust zijn en lopen op een accu. Een overzicht van de instellingen van de instrumentatie voor verschillende StarCount systemen word aan het eind van dit hoofdstuk gegeven.

In hoofdstuk 3, word het eerste systeem, de StarCount 1.0 besproken (1). Dit instrument was ontworpen om immuno-magnetisch geselecteerde cellen af te beelden en te tellen met behulp van een DNA kleurstof, Acridine Orange. We hebben de prestaties van dit systeem geëvalueerd door het bepalen van de concentraties van CD45+ leukocyt, CD3+ T lymfocyten en CD19+ B lymfocyten in menselijk bloed. Deze celpopulaties kunnen goed gedefinieerd worden door het gebruik van slechts één soort antilichaam. Door het vergelijken van cel tellingen verkregen met de SC1.0 met die van “single-platform” (SP) (TruCount) en “dual-platform” (DP) flow cytometrie methoden, hebben we de nauwkeurigheid van ons systeem beoordeeld. De resultaten hiervan zijn een uitstekende precisie en lineairiteit. In het geval van CD4+ cellen worden zowel CD4+ T lymfocyten als dimme CD4+ monocyten geselecteerd als men een immuno-magnetische labeling gebruikt die niet specifiek is voor één type subpopulatie. In dit geval moet een tweede immuno-magnetische labeling gebruikt worden om nauwkeurig de concentratie van CD4+ T lymfocyten te meten voor het bepalen van de HIV status van een patient.
De verwezenlijking van de SC 2.0, het eerste nauwkeurige systeem voor het bepalen van de concentratie van CD4+ T lymfocyten, word in hoofdstuk 4 besproken (2, 3). Dit systeem bepaalt de CD4+ T lymfocyten populatie als zijnde de CD3+CD4+ populatie. Eén antilichaam wordt gebruikt voor immuno-magnetische selectie en de andere voor immuno-fluorescente labeling. Op deze manier worden dimme CD4+ monocyten uitgesloten. Het feit dat de geselecteerde cellen positief zijn voor twee antilichamen geeft de mogelijkheid om twee verschillende labelings systemen te gebruiken: CD4-ferrofluid (CD4FF) / CD3-Phycoerythrin (CD3PE) en CD3FF/CD4PE. Beide systemen gaven goede resultaten en waren vergelijkbaar met elkaar en, nog belangrijker, met de twee flow cytometrie methoden (tetraCXP en TruCount) die vaak als gouden standaard worden gebruikt. Uitstekende precisie, nauwkeurigheid en lineairiteit van de SP ICM in de klinisch relevante concentraties van CD4+ T lymfocyten (0 – 500/µl) voor het volgen van HIV infecties werden gehaald.

Verdere ontwikkeling van de SC 2.0 leidde tot de SC 2.1, een systeem dat zowel de CD4 als de CD8 concentratie kan bepalen evenals de CD4/CD8 verhouding. Dit systeem is specifiek ontwikkeld voor het volgen van HIV infecties bij kinderen (Hoofdstuk 5). De sample bereiding is een 1-steps process waarbij het bloed immunologisch gelabeled word met CD3-magnetische nanodeeltjes, CD4-Phycoerythrin (PE) en CD8-Peridinin-Chlorophyll-Protein Complex (PerCP). De gevoeligheid van de SP ICM voor het gelijktijdig detecteren van CD8+ T lymfocyten en dimme CD8+ T lymfocyten werd onderzocht met behulp van PerCP magnetische bolletjes met verschillende intensiteiten. De invloed van overspraak van PE fluorescentie in het PerCP kanaal op de bepaling van de CD8 concentratie is ook besproken. Een systematisch te lage bepaling van de CD8 concentratie door het SC 2.1 systeem werd van te voren verwacht.

We hebben de CD4 en CD8 concentraties en de CD4/CD8 ratio vergeleken in bloed van 50 HIV negatieve volwassenen, 50 HIV positieve volwassenen en 45 HIV negatieve kinderen zoals bepaald door ons systeem (SP ICM) en een “single-platform” flow cytometer (tetaCXP). Voor al deze experimenten werd een goede correlatie behaald tussen deze twee systemen. De CD4 concentraties, zoals bepaald door ons systeem, waren vergelijkbaar met die bepaald door de flow cytometer, terwijl de CD8 concentratie die door ons systeem werd bepaald ongeveer 10% lager lag dan bij de flow cytometer. Deze te lage bepaling van de CD8 concentratie werd deels veroorzaakt door een klein percentage dimme
Samenvatting

CD8⁺ T lymfocyten (~10%) die of niet werden gedetecteerd door ons systeem of door overspraak van PE niet werden geteld.

Om tot een volledig automatisch systeem te komen dat ook gebruikt kan worden door minder getraind personeel, werden in dit onderzoek twee nieuwe beeld analyse algorithmen ontwikkeld, de dynamische methode en de maximale entropie methode. Beide methoden presteerden vergelijkbaar met een handmatige methode. De dynamische methode vertoonde een iets grotere spreiding dan de maximale entropie methode. Belangrijker is nog dat de dynamische methode veel tijd kost en daardoor minder geschikt is voor toepassing in een druk routine laboratorium. De maximale entropie methode was veelbelovend en is uitgebreid getest in een grote klinische trial in Bangkok (zie Hoofdstuk 7).

In hoofdstuk 6 presenteren we de ontwikkeling van een nieuwe immuno-cytochemische methode om de CD4 concentratie te bepalen. Deze methode combineerde immuno-magnetische selectie van CD4 positieve cellen met behulp van alleen het CD4 antilichaam met cel activatie om geselecteerde monocyt en granulocyten te onderscheiden van CD4⁺ T lymfocyten. Bloedmonsters werden behandeld met de DNA kleurstof Hoechst33342, de oxidatieve burst stimulator Phorbol 12-myristate 13-acetate en Dihydrorhodamine123. Door immuno-magnetische labeling met CD4 worden CD4⁺ T lymfocyten, dimme CD4⁺ monocyten en enkele niet specifiek gebonden granulocyten naar een analyse oppervlak gebracht uit een kamertje met een bekend volume. Twee fluorescentie plaatjes worden opgenomen en geanalyseerd: een Hoechst33342 plaatje (DNA plaatje) laat alle magnetisch geselecteerde leukocyten aan het oppervlak zien en het Rhodamine123 plaatje laat alle geactiveerde monocyt en granulocyten (hoofdzakelijk neutrofielen) zien. Door de verkregen aantallen cellen in beide plaatjes van elkaar af te trekken, kan de absolute CD4⁺ T lymfocyten concentratie bepaald worden. Het principe van dit systeem werd gecontroleerd met bloedmonsters van 29 gezonde donoren met behulp van een fluorescentie microscoop. Er werd geen verschil gevonden in activatie door oxidatieve burst tussen 42 HIV positieve patiënten en 18 gezonde donoren met behulp van flow cytometrie. Hierop werd een toegewijd SP ICM instrument gebouwd (SC 3.0) dat werd getest en getoetst door het vergelijken van de CD4⁺ T lymfocyten concentratie van 42 HIV positieve patiënten met die verkregen door flow cytometrie. Een redelijk goede correlatie werd behaald. Bland-Altman grafieken toonden aan dat de SP ICM methode vergelijkbaar was met flow cytometrie methoden.
Samenvatting

In hoofdstuk 7 worden de resultaten gepresenteerd van een veldtest van het SC2.1 systeem met HIV geïnfecteerde patienten dat werd uitgevoerd in het Siriraj ziekenhuis in Bangkok, Thailand. CD4 en CD8 concentraties en CD4/CD8 verhoudingen van bloedmonster van 460 HIV positieve volwassenen en 47 HIV positieve kinderen werden verkregen met behulp van de SC 2.1. De maximale entropie threshold methode werd toegepast. De resultaten werden respectievelijk vergeleken met een SP FCM methode (FACSCount) en een “dual-platform” (DP) FCM methode (FACSCan). CD4 concentraties verkregen met de SC 2.1 waren vergelijkbaar met die van de FACSCount en de FACSCan met een bias van respectievelijk 9.1% en 11.2%. Bij zeer lage CD4 concentraties ($\leq 50/\mu l$) waren er enkele gevallen waar de FACSCan en de SP ICM een duidelijk hogere concentratie gaven als we de FACSCount als gouden standaard gebruiken. Deze te hoge bepaling zal waarschijnlijk niet het maken van klinische beslissingen of het volgen van de HIV status beïnvloeden, zolang altijd dezelfde Methode wordt gebruikt. De CD8 concentratie zoals bepaald door de SP ICM laat een consistente te lage waarde zien in vergelijking met de FACSCount, vergelijkbaar met dat wat eerder in hoofdstuk 5 werd gevonden. Uit deze grote klinische trial concludeerden we dat een volledig geautomatiseerde SP ICM kan worden gemaakt door de maximale entropie methode in de analyse software te implementeren. De SP ICM zal dan een erg goed alternatief zijn voor de dure SP en DP FCM methoden in point-of-care omstandigheden in arme landen.

In hoofdstuk 8 presenteren we het principe van een nieuwe technologie voor het opbouwen van een CD3+ T lymfocyten immobilisatie platform voor het bepalen van CD4 en CD8 concentraties. Complex biostructuren werden gemaakt waarin streptavidine (SAV) is gebonden aan β-Cyclodextrin (βCD) zelf-geassembleerde monolagen (SAMs) op een silicium oxide oppervlak. CD3 monoclonale antilichamen (Mabs) werden verbonden aan de βCD SAMs met behulp van SAV om zo een CD3 cel immobilisatie platform te vormen. Dot-blot experimenten toonden aan dat biotin-protein G (biotin-PG) voldoet als Fc receptor voor de CD3 monoclonale antilichamen (B-B12 of biotin-CRIS-7) om de CD3 MAb lagen op te bouwen. Surface Plasmon Resonatie (SPR) sensogrammen lieten een succesvolle stapsgewijze opbouw van een biotin-CRIS-7 en een B-B12 laag op de βCD SAMs zien in twee verschillende schema’s. Een eerste experiment met cellen werd uitgevoerd om de capaciteit van de B-B12 laag om CD3+ T lymfocyten te vangen te onderzoeken. De CD3+ T lymfocyten, de CD3+CD4+ T lymfocyten en de CD3+CD8+ T
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lymfocyten concentraties werden onderzocht en de verhouding tussen het aantal cellen dat werd geïntroduceerd en het aantal cellen dat werd geteld werd bestudeerd. De resultaten van deze eerste experimenten met cellen wijzen erop dat de B-B12 lagen die werden onderzocht geschikt zijn voor het bepalen van CD4 en CD8 concentraties. Om een definitiere conclusie te trekken zijn verdere experimenten noodzakelijk. Wij verwachten dat een nieuwe methode kan worden ontwikkeld om CD4 en CD8 concentraties te bepalen gebaseerd op de “cel-vangende” Mab laag die aan βCD SAMs (30 - 33) is gekoppeld in combinatie met onze SP ICM (1 - 3).

Concluderend kunnen we stellen dat de SP ICM die wij hebben ontwikkeld een goed systeem is om de CD4 en CD8 concentraties te bepalen voor HIV stagering in point-of-care omstandigheden in arme landen.
艾滋病于1981年在洛杉矶被确认为一种病毒感染，从那时起，这种病毒感染已经成为一个全球性的健康问题。在许多国家，特别是在一些落后的发展中国家，这种病毒感染甚至已经开始导致了严重的毁灭性危机。近年来，科学家们在研究艾滋病病毒感染机制，以及针对艾滋病病毒的抗反转录病毒治疗方面取得了一系列卓有成效的成果。值得一提的是，针对艾滋病病毒的抗反转录病毒治疗已经是一种广泛采用的方法，治疗的花费也变得越来越低。这对于身处落后的发展中国家的病人来说，无疑是一个福音。

艾滋病病毒感染的病理特点决定了抗反转录病毒治疗需要在适当的时间进行。CD4-T淋巴细胞计数是最重要的确定疾病阶段和发展的临床参数。当感染艾滋病病毒后，患者血液中的CD4-T淋巴细胞计数会下降。在临床上，当艾滋病病毒感染者血液中的CD4-T淋巴细胞下降到每微升低于200时，患者被称为艾滋病患者，只有当患者的CD4-T淋巴细胞数可及的情况下，抗反转录病毒治疗才可能有效的进行。同时，CD4-T淋巴细胞计数对于决定何时开始用药，何时改变用药，以及评估治疗效果等方面，都是至关重要的。对儿童艾滋病病毒感染监控，CD4-T淋巴细胞数在总淋巴细胞数中的百分比和CD4/CD8淋巴细胞数比率也是必需的。

流式细胞仪方法是测量CD4-T淋巴细胞数和CD8-T淋巴细胞数的黄金标准方法。但是，仪器耗资十几万到几十万美元，每样品测试费用几十美元。仪器需要专业技术人员操作，并需要稳定的电源，以及精心的维护和保养。对一些落后的发展中国家，它是一种昂贵而不能普及的方法。因此，价格低廉而又能够准确测量出CD4-T淋巴细胞数的实验方法，以及可以产业化，同时使用简单而又可以普及化的仪器，对于人类赢得时间攻克艾滋病难题，对于可能或者已经被艾滋病病毒感染困扰的发展中国家来说，其重要意义和紧迫性不言而喻。
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我们设计，测试，和优化了一系列磁性选择和荧光标记目标细胞的免疫生物学方法，并在此基础上开发和测试了一系列自动化的手术细胞仪（SP ICM）。它们价格低廉，操作方法简单，能够迅速而准确地测量血清中的 CD4-T 淋巴细胞数和 CD8-T 淋巴细胞数。该系列的样品仪器，曾名为 EasyCount，现名为 StarCount 系列。本论文阐述了如上研究成果。

章节 1 陈述了背景知识：人类的血液，爱滋病毒感染和 CD4-T 淋巴细胞数削减的关系，爱滋病毒感染和爱滋病危机的全球性状况以及免疫监控 CD4-T 淋巴细胞数的重要性。

章节 2 介绍了我们系统的免疫生物学方法和仪器的组成。以 StarCount 1.0（曾名为 EasyCount）为例，该系统的细胞计数方法是建立在免疫磁性选择和荧光标记相结合的基础上的。具体来说，载有抗体（CD45，CD3，CD19，CD）是一种特定的表达细胞表面抗原的分类方法。的磁性纳米微粒和血液中的 CD45 白血球，CD3-T 淋巴细胞，和 CD19-B 淋巴细胞能够通过抗原-抗体相互作用的方式结合，结合了磁性微粒的血细胞在磁场中定向移动到样品槽表面。由于这些细胞中的脱氧核糖核酸 (DNA) 先被一种核酸染料叮凝固荧光标记，它们就可以被荧光图象细胞仪识别，并被分别计数。在仪器方面，我们应用，设计和组装了小巧而高效的磁场系统，低廉而高效的激发荧光标记的照明系统，和简单实用的荧光影像光学系统。同时，样品仪器带有 CCD 照相机，单板计算机和触摸屏幕。细胞的计数由我们自己开发的图像分析算法软件执行，计数过程自动，迅速，准确。

为了在发展中国家普及，我们的仪器具备了价格低廉（大约三千美元），使用方便，无需特别培训操作人员，样品检测费用低（大约每样品测试三美元），便携，以及能够在使用充电电池的情况下正常工作等特点。我们在血液样本稀释倍数，试剂用量，试剂培养时间，和磁性分离时间的确定方面，在样品槽和 DNA 荧光标记物，免疫荧光标记物，免疫磁性微粒类型的选择方面做了细致的研究。最终优化成为现行的方法：用量少，样品处理简单，测试费用低。

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StarCount 1.0（章节 3）被用来测试 CD45 白血球，CD3-T 淋巴细胞，和 CD19-B 淋巴细胞。StarCount 1.0 与黄金标准方法例如单一封闭式细胞仪方法（TruCount）和双重平台式流式细胞仪方法相比较，结果显示 StarCount 1.0 系统的优秀的精确性、高敏感性，和正确的选择性。

StarCount 1.0 系统对于实现 CD4-T 淋巴细胞计数提供了一个坚实的基准。CD4-T 淋巴细胞计数是一个公认的关键。一直以来，并没有一个价格低廉，简单易行，又准确可信，适用于发展中国家的计数方法。其难点在于，CD4-T 淋巴细胞并不是唯一的表面有 CD4 抗原的血细胞。因此，其计数会不可避免的受到干扰。例如，CD4 单核细胞，和少量粒细胞（依靠非特异性结合）也会同时被 CD4 免疫磁性方法选择，并被计入 CD4-T 淋巴细胞数。

针对这个问题，我们创造性的采用了几种不同的免疫学和细胞学方法，成功的利用我们的系统实现了准确的 CD4-T 淋巴细胞计数系统。下面分别简单介绍这几种方法：

1. CD4-T 淋巴细胞表面同时有 CD3 和 CD4 抗原。利用这个特性，我们引入了 CD3 和 CD4 双抗体选择机制：一个抗体用于免疫磁性选择，另一个用于免疫荧光标记。这样，其它的表面有 CD4 抗原的细胞就被成功的排除在我们的细胞计数之外。通过研究我们发现，CD4-磁性微粒（CD4FF）/CD3 流红素荧光标记（CD3PE）或者 CD3FF/CD4PE 两种方法得到了可彼此互换的好结果。第二种方法为日后发展 CD4 和 CD8 同时计数系统提供了可能性。更重要的是，小型临床测试证实我们的单平台平台仪象细胞仪，星 acute StarCount 2.0 系统（章节 4），可与黄金标准方法例如单一封闭式细胞仪方法（TruCount）和（tetraCXP）交换。在为监控 HIV 感染的 CD4-T 淋巴细胞数的重要的临床范围内（0 ~ 500/μl），我们的系统达到了优秀的精确度、准确性和线性。

2. 在 StarCount 2.0 系统的基础上，我们进一步发展并构建了特别用于监控儿童 HIV 病毒感染的 StarCount 2.1 系统（章节 5）。该系统利用 CD3-磁性微粒（CD3FF），CD4 流红素荧光标记（CD3PE）和 CD8 荧光标记（PerCP），同时计数 CD4-T 淋巴细胞和 CD8-T 淋巴细胞，以及确定 CD4/CD8 比率。我们分别利用 StarCount 2.1
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系统和黄金标准方法（单平台流式细胞仪 tetraCXP 方法）去分析 50 例 HIV 阳性成人，50 例 HIV 阳性成人和 45 例 HIV 阳性儿童的血样，两种方法得到的 CD4+ T 淋巴细胞计数，CD8-T 淋巴细胞计数，和 CD4/CD8 比率具有优秀的互换性。

为了实现细胞计数系统的智能化和完全自动化，我们开发和测试了两种新的自动图像分析算法：动态域值算法和最大熵域值算法。两个完全自动化的图像分析算法可以替代经过训练的操作员手工确定域值的方法。特别值得一提的是，最大熵域值算法由于计算迅速准确，很适合用于测试大量血液样品。它在泰国曼谷进行的临床测试中被采用。

StarCount 2.1 系统在泰国 曼谷 Siriraj 医院的测试中取得了良好的结果（章节 7）。460 例 HIV 阳性成人和 47 例 HIV 阳性儿童的全血中的 CD4, CD8 计数和 CD4/CD8 比率被测定。最大熵域值算法也首次被用来处理分析大量细胞图像。获得的结果与由单平台流式细胞仪方法（FACScan）和由单平台流式细胞仪方法 (FACSCount) 获得的结果可比性优(R = 0.97)。对于 CD8 计数，相对于 FACSCount 方法，我们的系统显示固定的大约 15% 的低估，这与我们在荷兰 MST 医院实验室里得到的结果一致。我们认为，我们的系统可以代替昂贵的单平台双平台流式细胞仪方法，从而普遍应用在资源匮乏的发展中国家。

3. 我们开发了一项新颖的用于 CD4 计数的免疫细胞化学方法（章节 6），该方法利用单核细胞，淋巴细胞独特的细胞氧化激活作用，把这些有可能对 CD4-T 淋巴细胞计数产生干扰的细胞区分开来；因此，可以用单一 CD4 抗体来进行 CD4-T 淋巴细胞的免疫磁性选择和计数。这样，单次血液样品检测的成本大大降低。同时，我们还用流式细胞仪方法分析了健康人和艾滋病感染者 的细胞氧化激活动力学过程。实验结果，这两种人群的单核细胞，淋巴细胞在细胞氧化激活作用方面没有明显区别。由此，利用细胞氧化激活作用来把单核细胞，淋巴细胞和 CD4-T 淋巴细胞区分开来这一原理的可行性被证实。在此基础上，我们制造并测试了新的图像分析仪 StarCount 3.0，对 42 例 HIV 阳性患者 CD4-T 淋巴细胞数的计数，以及与流式细胞仪
方法得到结果的比较表明，StarCount 3.0 和流式细胞仪方法也具有优秀的可比性和可互换性。

4. 我们与 David N. Reinhoudt 的超分子化学技术小组合作。利用分子自组装技术，成功的在氧化硅表面形成了一个复杂的生物结构。在这里，β-Cyclodextrin (β-环糊精) 先以单分子层形式自组装在氧化硅表面；然后，streptavidin (SAV) 通过分子间作用力，覆盖在 β-环糊精分子层上；接着，连接 biotin (生物素) 的 CD3 抗体再通过 biotin (生物素)-SAV 的相互作用力附着在最外表面，形成 CD3 细胞选择平台（章节 8）。点杂交实验表明，生物素蛋白 G (biotin-protein G) 很适合作为 CD3 抗体(MAb) (B-B12 或 biotin-CRIS 7) 的 Fc 受体来组建 CD3 抗体的平台。而且，CD3 抗体的取向有利于和细胞表面抗原结合。表面等离子共振(SPR) 实验显示了逐步自组装的动力学过程。该表面可以用于选择吸附并固定 CD3-T 淋巴细胞，进而利用免疫荧光染色的方法分别计数 CD3-T 淋巴细胞中的 CD4-T 和 CD8-T 淋巴细胞。细胞实验成功地验证了 CD3 细胞选择平台对于 CD3-T 淋巴细胞的选择；而对于 CD3-T 淋巴细胞，CD3CD4-T 淋巴细胞和 CD3CD8-T 淋巴细胞的计数表明，被计数的细胞的数量与实验投入的细胞的数量成线性关系。我们预计，利用这项组建 CD3 抗体平台的技术，结合我们的单一平台图像细胞仪(SPICM) 的免疫荧光标记方法，我们可以发明新一代的微型单平台图像细胞仪。由于该系统的的特异选择性，和使用微量样品的特点，其成本将大幅度降低，而操作的普及性和简单性也将大大提高。从更高的角度看，该技术可以应用于构建微型细胞分析仪，高通量细胞分析仪，和研究细胞对于各种刺激的反应的筛选设备。

我们发展的单一平台图像细胞仪系统能够替代昂贵的单平台或双平台流式细胞仪方法。我们相信，在不远的将来这个系统将被广泛的应用于发展中国家的艾滋病感染监控。
Acknowledgements

It is a Monday morning in the early December. I am sitting in the cozy office, outside a storm was just whistling away. Suddenly, a giant rainbow appears in front of my window, touchable. It is beautiful! Glaring at the rainbow, I feel it is a bridge: Wandering on it, I could look through the past years in BPE group and in Enschede.

“Well”, I say to myself, “it is the time to thank my dear colleagues, friends and family.”

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Xiao Li (Lily) 欣晓
Publications

PUBLICATIONS

Publications in Peer-reviewed Journals:


Conference Contributions:

1. Li X, Yneti A, Lunter B, Breukers C, Greve J, Tibbe AGJ, Terstappen LWMM. Affordable cell enumeration for HIV staging. ISAC XXIII International Congress, Québec City, Canada, 2006. (X Li was nominated as one of the five Exceptional Student Award Finalists and gave an oral presentation for the competition of the winner.) (Oral presentation and Poster)


Curriculum Vitae

Xiao Li was born on March the 5th, 1976 in HuangShan, Anhui, P.R. China. In 1994, she started her study in Institute of Life Sciences, University of Science and Technology of China (USTC), majoring in Molecular Biology and Cell Biology. After graduation, she joined in National Public Health Institute, Department in Turku, Finland in October 1999. She was involved in the research on the pathogenesis of Reactive Arthritis. Her major research focused on “Acidification of Salmonella-containing phagosomes in the HLA-B27 transfected human monocytic U937 cells”. In April 2002, she came to Biophysical Engineering group, University of Twente in Enschede, the Netherlands, and worked as a researcher. In April 2004, she started her Ph.D. project “AIDS in Africa: Affordable HIV Staging” under the supervision of Professor Jan Greve and Professor Leon WMM Terstappen. Her PhD work concerns the development of an affordable image cytometer system for CD4 enumeration that is urgently needed in resource-constrained countries. During her PhD studies, she has co-authored 11 papers, which are (will be) published in well-recognized scientific journals, given 3 oral presentations and contributed 9 posters in International and National Congresses. In 2006, she was nominated as one of the five Exceptional Student Award Finalists on ISAC XXIII International Congress, Québec City, Canada.
Color Figures

FIG. 1. G, H. (Page 50, in Chapter 3)

FIG. 4. (Page 102, in Chapter 5)
FIG. 1. (Page 125, in Chapter 6)

FIG. 8. (Page 131, in Chapter 6)

FIG. 5. (Page 173, in Chapter 8)
FIG. 3. (Page 154, Chapter 7)