Development of WHO Guideline-Complying CD4 Diagnostic Chip

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Abstract: Accurate HIV diagnosis using current WHO complying diagnostic chips that measure CD4 protein expression faces challenges due to donor variability in expression levels and difficulties in isolating target cells with high purity. To overcome these limitations, quantitative measurement of $CD4^+$ and $CD8^+$ T cells, along with the effective removal of red blood cells (RBCs) and granulocytes, is essential. We present a self-powered CD4 diagnostic chip platform designed for high0purity target cell separation using a small volume of whole blood. The CD4 diagnostic chip employs magnetic separation to remove non-target cells through negative selection, utilizing ferromagnetic particle-filled inlets and a magnet positioned beneath the inlet. Excessive RBCs and target cells are further separated by size using a microfluidic lattice. This rapid separation process, facilitated by a degassed polydimethylsiloxane (PDMS) chip, achieves efficient target cell isolation within 20 minutes. During separation, the LEGO-integrated smartphone microscope records a real-time video, which is analyzed using Python-based code. The code distinguishes and removes excess granulocytes based on pixel intensity and precisely counts pure target cells, enabling analysis and potential diagnosis within 1 minute post-separation. The cD4 diagnostic chip is a simple, precise, and rapid platform requiring minimal blood volume, compliant with WHO guidelines for HIV diagnosis.

1 INTRODUCTION

Detection of CD4 expression from whole blood is critical technology for the clinical diagnosis of human immunodeficiency virus (HIV). Curren diagnostic methods that comply with World Health Organization (WHO) guidelines, such as the 'VISITECT CD4' assay, typically involve lysing whole blood and measuring the total CD4 protein expression (Lechiile et al., 2022). However, these approaches have significant limitations.

CD4 protein expression levels on $CD4^+$ T cells exhibit significant interindividual variability. As a result, individuals with lower CD4 expression can be misdiagnosed, despite having $CD4^+$ T cell counts within the normal range. Furthermore, CD4 expression is not restricted to T lymphocytes; monocytes also express CD4, potentially confounding diagnostic accuracy. Additionally, when whole blood is processed without lysis and traditional microfluidic methods are used to isolate target T cells, sample purity issues may arise, further affecting diagnostic reliability. Positive selection methods for $CD4^+$ T cell isolation inadvertently include CD4-expressing monocytes, as previously described, whereas negative selection methods, while yielding higher purity, may co-isolate excess granulocytes, given their higher relative abundance in whole blood.

To address these limitations, it is crucial to rely on quantitative $CD4^+$ T cell counts, rather than CD4 expression levels, for unbiased assessments. Quantitative measurements for HIV diagnosis require a $CD4^+$ T cell concentration under 200 cells/μL and a CD4/CD8 T cell ratio below 1. However, existing studies on quantitative CD4+ T cell assessment do not consistently comply with WHO guidelines (Yeh et al., 2017).

To meet WHO standards, diagnostic platforms must achieve detection at concentrations as low as 200 cells/μL using a minimum whole blood sample volume of 100 μL and involve a preparation-to-assay time under 5 minutes.

Here, we developed a CD4 diagnostic chip for a rapid, precise, and simplified HIV diagnostic platform suitable for point-of-care testing (POCT). Requiring only 5 μL of whole blood, the CD4 diagnostic chip detects concentrations as low as 100 cells/μL. Using a smartphone microscope integrated with a LEGO-based setup, real-time video capture and Python-based software enable quantitative cell counting at the target cell outlet. The distinct pixel intensity differences between granulocytes and lymphocytes

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facilitate visual and software-based cell type differentiation. Our three-step separation process yields high-purity $CD4^+$ and $CD8\ T^+$ cells with quantitative counts in under 25 minutes, meeting high precision and purity requirements with Python-based data analysis.

2 METHODS

2.1 Design and Development of the Lego-Integrated Smartphone Microscope

Lego bricks were utilized as a structural framework to stabilize the various components of the setup. A smartphone (iPhone SE) was mounted in an inverted position and aligned with the 10X eyepiece lens. A mirror was used to connect the 20X objective lens to the eyepiece lens. An LED light source was positioned at the top of the Lego structure. The CD4 diagnostic chips were securely placed on a mechanical stage integrated into the framework.

2.2 Fabrication of the CD4 Diagnostic Chip

The CD4 diagnostic chip was designed following previously reported methods (Shin et al., 2023). The chip was fabricated using standard SU-8 photolithography and polydimethylsiloxane (PDMS) molding techniques. To assemble the chip, it was positioned on top of a cover glass and affixed. The assembled chips were then stored in an oven at 65°C for future use.

2.3 CD4 Diagnostic Chip Procedure

The entire chip was degassed for 20 minutes in a vacuum chamber, with the outlet reservoirs covered by cover glass. For long-term storage and portable use, the chip could be packaged in an aluminum vacuum seal (yeh et al., 2017). Whole blood was collected in 10 mL BD EDTA Vacutainer tubes, then diluted 1:10 in 0.1% BSA. The blood was mixed with either a human $CD4^+$ T cell isolation kit or a human $CD8^+$ T cell isolation kit and incubated for 15 minutes at 4°C. Upon removal of the chip from the chamber, 40 µL of the blood mixture and 100 µL of 0.1% BSA buffer were pipetted into the respective blood and buffer inlets. To assess the purity, precision, and recovery of the target cells, CD45 (FITC) and either CD4 (APC) or CD8 (APC) antibodies were added to 100 µL of 0.1% BSA before being loaded onto the chip. After 30 minutes, the target cell outlet was imaged using a fluorescence microscope (Ti2, Nikon) equipped with a motorized stage and a scientific CMOS camera (Andor Inc.).

2.4 Characterization of the Cell Separation Process

Whole blood samples were serially diluted from 1X to 1/16X concentrations, and cell separations were performed using CD4 diagnostic chips. All processes were recorded in slow-motion mode using a smartphone, with video frames focused on the region immediately preceding the target outlet. Post-capture, cell identification and quantification were conducted using Python-based code.

3 RESULTS

3.1 Characterization of Target Cell Separation in the CD4 Diagnostic Chip

To minimize cost and simplify construction, we designed an optical setup using a smartphone, integrated with a modular LEGO-based framework (Fig. 1A). The CD4 diagnostic chip is positioned on the objective lens and fixed with a mechanical stage, enabling cell imaging via smartphone as they flow through the device. Target cells are isolated by loading 40µL of whole blood sample and 100µL of 0.1% BSA buffer into the blood and buffer inlets, respectively.

We further enabled detection of the marker of the target cells separated from the CD4 diagnostic chip by imaging the chip's outlet. This was achieved by adding a fluorescent antibody to the 0.1% BSA buffer prior to loading it onto the chip (Fig. 1B). Non-target cells in the blood sample are magnetically labeled with a negative selection kit reagent. Upon loading the blood sample, target cells and non-labeled red blood cells (RBCs) pass through the microfluidic lattice, while labeled non-target cells are trapped in the blood inlet, which contains ferromagnetic particles and a magnet positioned beneath the blood inlet. The RBCs are directed to a waste outlet, while target cells are washed with buffer and sorted into the target outlet (Fig. 1C). Measurement of $CD4^+$ and $CD8^+$ T cell separation throughput over time revealed a consistent decline after sample loading. This is because of the gradual reduction in negative pressure over time during the

PDMS degassing process (Fig. 1D).

We then evaluated the effectiveness of target cell separation from whole blood by assessing the purity and precision of the sorted cells. Purity was calculated as the ratio of target cells to the total cell count in the target cell outlet, while precision was calculated as the ratio of target cells in the target cell outlet to the total number of target cells across both outlets. As a result, we achieved successful separation using CD4 diagnostic chips, obtaining an average purity of 91.7% and precision of 86.2% for $CD4^+$ cells, and a purity of 86.2% and precision of 84.8% for $CD8^+$ cells, all within 30 minutes (Fig. 1E). Additionally, we assessed the recovery rates of target cells, which were 27.0% for $CD4^+$ T cells and 21.7% for $CD8^+$ T cells (Fig. 1F). Recovery was calculated as the ratio of target cells collected in the target cell outlet to the total number of target cells loaded at the inlet. Because of the vacuum pump driven system, the separation throughput gradually decreased over time. However, throughput could be enhanced by simply reducing the volume of the blood sample.

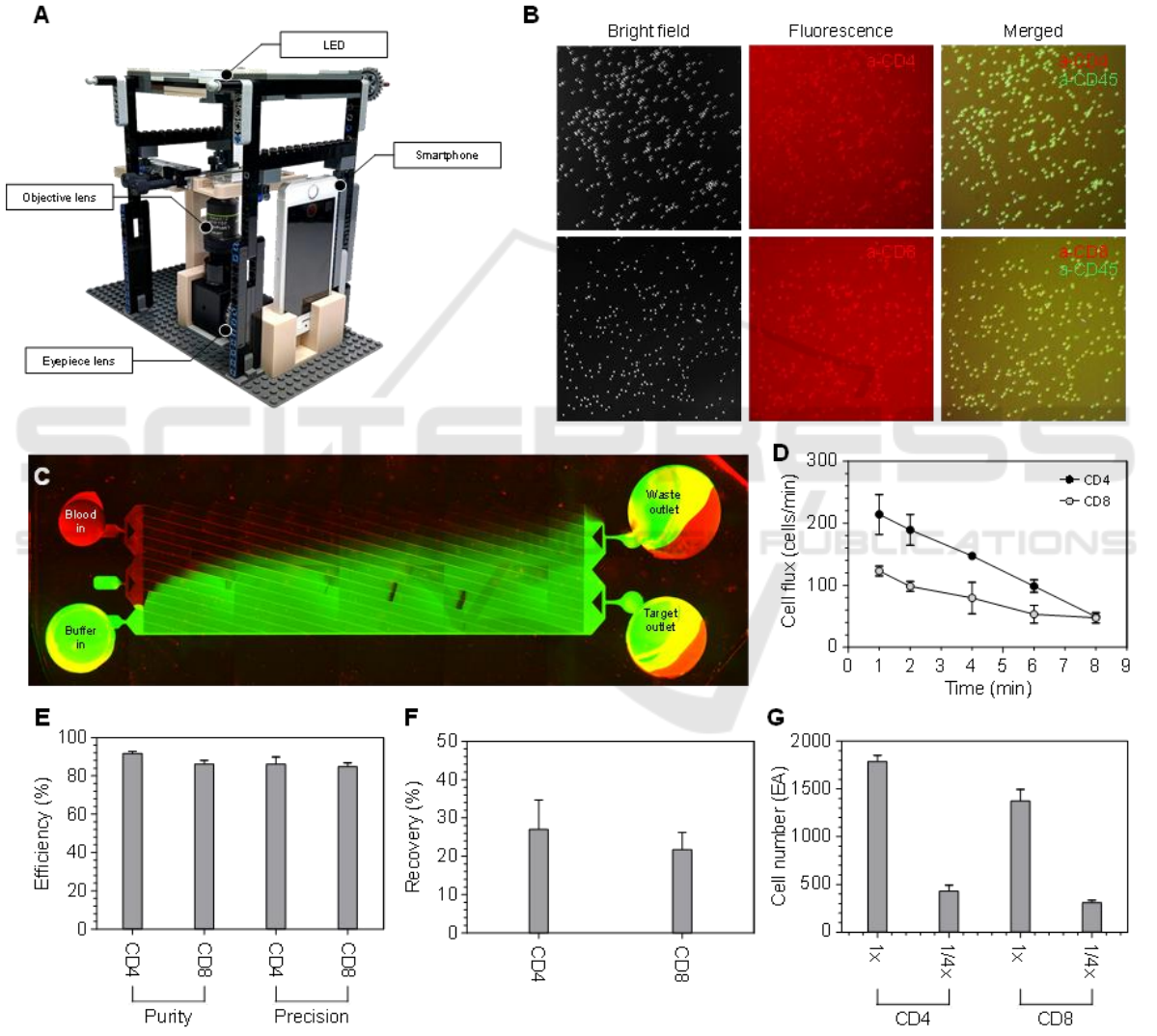

Figure 1: Separation of $CD4^+$ and $CD8^+$ T cell using the CD4 diagnostic chip. (A) Image of the smartphone microscope setup with Lego-integrated components. (B) Fluorescence images of sorted $CD4^+$ and $CD8^+$ T cell. (C) Fluorescence image depicting microfluidic washing process. (D) Throughput of $CD4^+$ and $CD8^+$ T cell separation flux over time. (E) Purity and precision of sorted $CD4^+$ and $CD8^+$ T cell. (F) Recovery rates of sorted $CD4^+$ and $CD8^+$ T cell. (H) Repeatability analysis of the CD4 diagnostic chip for separating $CD4^+$ and $CD8^+$ T cell.

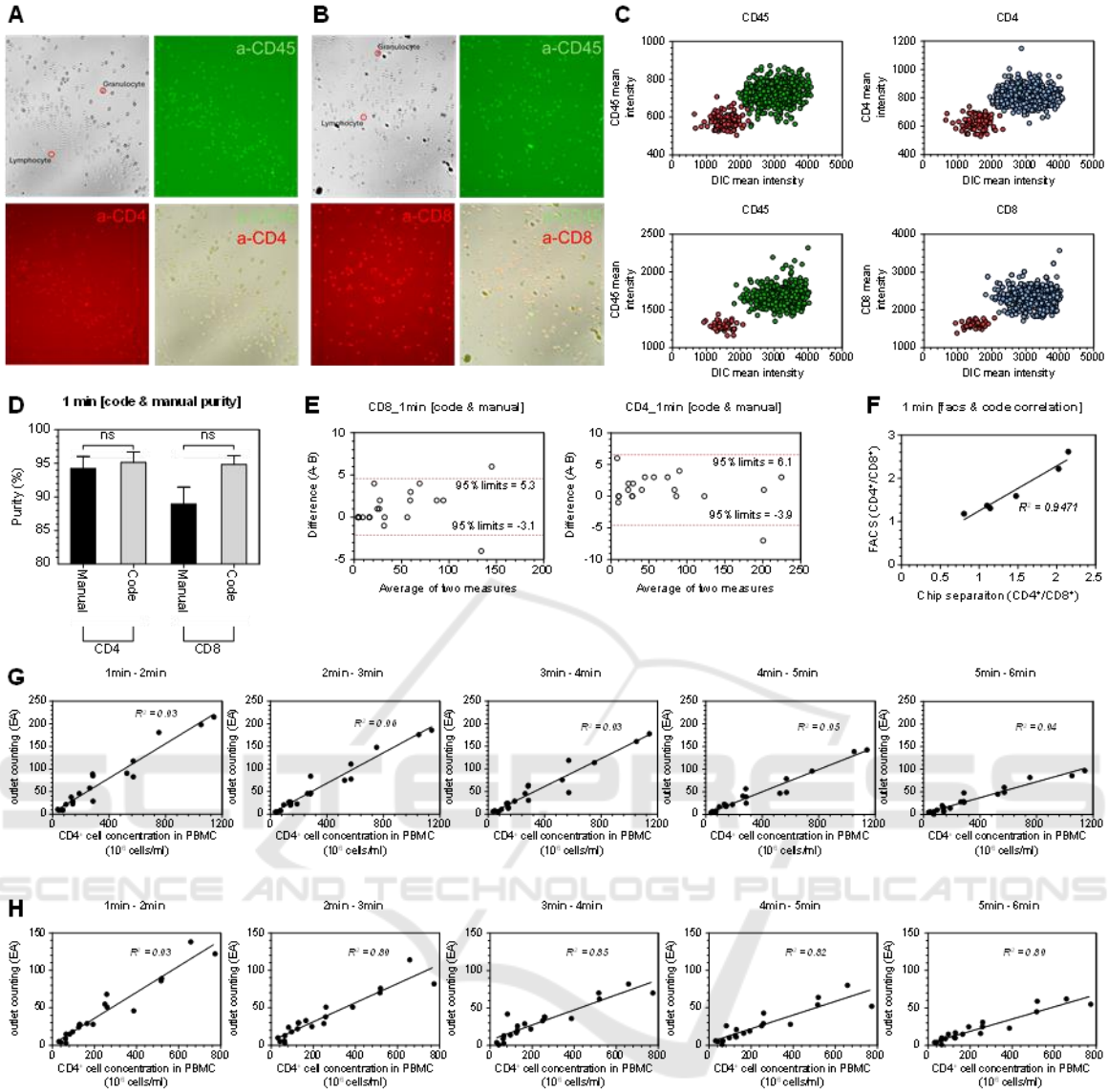

Figure 2: Quantification of CD4$^+$ and CD8$^+$ T cell following separation via the CD4 diagnostic chip. (A) Fluorescence imaging of sorted CD4$^+$ T cell and (B) CD8 T$^+$ cell, showing residual granulocytes. (C) Plot of pixel intensity differences distinguishing cell types. (D) Comparison of CD4$^+$ and CD8$^+$ T cell purity assessed via Python-based automated counting versus manual counting over a 1-minute video segment. (E) Evaluation of Python code and manual counting methods. (F)Ratio comparison of CD4$^+$ and CD8$^+$ T cell between Python-based automated code counting and FACS analysis. (G) Quantitative counts of CD4$^+$ T cells and (H) CD8$^+$ T cells obtained from serially diluted whole blood samples, as analyzed by Python code.

We also investigated the repeatability of the CD4 diagnostic chips to assess potential performance variability. Utilizing whole blood samples from the same donor, we processed undiluted (1X) and 1/4X diluted whole blood supplemented with 0.1% BSA and quantified the CD4$^+$ and CD8$^+$ cell counts at the garget cell outlet (Fig. 1G). The results demonstrated no significant differences in chip performance across measurements, indicating high repeatability.

3.2 Detection of Target Cell with Python-Based Code in the CD4 Diagnostic Chip

To detect CD4$^+$ T cells at concentrations below 100 cells/μL, it is essential to achieve precise separation, eliminating non-target cells entirely. However, some granulocytes persist in the target outlets alongside CD4$^+$ and CD8$^+$ T cell (Fig. 2A and 2B).

Given that target cells are isolated using a negative selection kit, donor variability influences cell separation outcomes. Although the purity of $CD4^+$ and $CD8^+$ T cell approaches 90%, the remaining 10% impurity arises from non-target cells that are not fully eliminated. In donors with a high abundance of granulocytes or reduced expression of granulocyte-specific markers, these granulocytes are inadvertently collected in the target cell fraction, thereby increasing the impurity. Notably, brightfield imaging reveals a significant difference between lymphocytes and granulocytes. Granulocytes exhibit lower pixel intensity due to granules, appearing as dark spots, in contrast to the more transparent appearance of lymphocytes (Fig. 2C).

The Python-based code, capable of distinguishing lymphocytes from granulocytes based on pixel intensity, was utilized to analyze 1-minute segmented videos recorded via smartphone to assess the purity of $CD4^+$ and $CD8^+$ T cell separation. A comparison between manual counting and Python-based automated counting methods revealed no significant differences between the two approached (Fig. 2D). To further evaluate the precision of cell counting, a comparative analysis of manual and automated methods was conducted, demonstrating that the Python-based code provides accurate and reliable counts, with no significant discrepancies observed (Fig. 2E). Given the clinical importance of the $CD4^+$/ $CD8^+$ T cell ratio for diagnosing HIV patients, we compared the Python-based automated counting method to conventional FACS analysis. The high correlation (R^2=0.95) between the two methods indicates strong agreement and confirms the reliability of the automated approach (Fig. 2F).

To assess the performance of the CD4 diagnostic chip, whole blood was serially diluted from 1X to 1/16X. Videos were analyzed using the Python-based code, with recordings segmented into 1-minute intervals starting from the initiation of the separation process. We observed a strong correlation between the concentration of $CD4^+$ T cells in PBMCs and the number of target cells counted using the automated method, yielding and R^2 value of 0.93 (Fig. 2G). Similarly, the concentration of $CD8^+$ T cells in PBMCs showed a high correlation with the automated cell count, also with an R^2 value of 0.93 (Fig. 2H).

4 DISCUSSION

We developed a CD4 diagnostic chip integrated with a LEGO-based smartphone microscope platform, offering a cost-effective, precise, and straightforward solution for target T cell separation. The Python-based code enables accurate counting of target cells at concentrations below 100 cells/μL, using a minimal blood volume of just 5 μL. The entire separation and counting process is completed within 30minutes, making it well-suited for point-of-care (POC) applications.

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