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Letters to *Analytical Chemistry*

Microfluidic CD4+ T-Cell Counting Device Using Chemiluminescence-Based Detection

Zuankai Wang,[†,‡] Sau Yin Chin,[†] Curtis D. Chin,[†] John Sarik,[§] Maritza Harper,[†] Jessica Justman,[||] and Samuel K. Sia*[,†]

Department of Biomedical Engineering, Columbia University, 351 Engineering Terrace, 1210 Amsterdam Avenue, New York, New York 10027, Department of Electrical Engineering, Columbia University, 1300 South West Mudd 500, West 120th Street, New York, New York 10027, and International Center for AIDS Care and Treatment Programs, Mailman School of Public Health, Columbia University, New York, New York 10032

This letter demonstrates a microfluidic platform for enumerating CD4+ T-lymphocytes from whole blood using chemiluminescence as a detection method. We microfabricated traps in a chamber and coated them with anti-CD4 antibody to isolate CD4+ T-cells. Based on cell surface-bound CD3 antibodies conjugated with horseradish peroxidase, incubation with chemiluminescent substrate produced a current in the photodetector that was proportional to the number of captured CD4+ T-cells. Analyzing 3 μL of whole blood, the platform exhibited high cell-capture efficiency and produced cell counts with high correlation to results obtained from flow cytometry. Compared to other lab-on-a-chip methods for CD4 counting, this method uses an instrument that requires no external light source and no image processing to produce a digitally displayed result only seconds after running the test.

For management of human immunodeficiency virus/acquired immune deficiency syndrome (HIV/AIDS), which remains a major pandemic in both developed[1] and developing countries,[2−5] there exists an unmet need for a point-of-care (POC) diagnostic device for determining the numbers of CD4+ T-cells from human whole blood samples. Clinically, a CD4 count below 200 cells per microliter of blood indicates a weak immune system and the onset of AIDS.[6] As such, current clinical guidelines from the World Health Organization for monitoring HIV-infected patients in places with poor medical infrastructure are based on clinical symptoms and absolute CD4 counts.[7] In addition to identifying the onset of AIDS in HIV-infected patients, CD4 counting is also useful for monitoring the immune system in patients undergoing antiretroviral therapy in order to direct changes in the drug regimen. Indeed, regular monitoring of the immune system will become increasingly important as antiretroviral therapy becomes available to a greater population of AIDS patients and as cases of drug resistance become increasingly prevalent.[8]

Currently, the gold standard for CD4 counting is flow cytometry. Despite its accuracy and high throughput, flow cytometry is not available in remote resource-limited settings due to its needs for laboratory infrastructure and a trained operator, as well as high cost. Availability of a low-cost and accessible CD4 counting device would produce a tremendous clinical impact via timely diagnosis of AIDS patients.[9−11] A recent public health consortium has suggested that a clinically useful POC CD4 test should, at minimum, be semiquantitative (with a minimum cutoff of 250 cells/μL), use finger prick blood or other nonvenous blood samples, be simple to perform, operate in less than 30 min from patient to result, and provide a simple read out.

While recent work has made progress toward this goal,[12−16] a low-cost device for remote POC CD4 counting is still lacking in the market.[9,10,17] This gap is partially caused by the technological challenges associated with CD4 counting, including a need for efficient CD4 isolation (hundreds of CD4+ T-cells out of millions

* Corresponding author. E-mail: ss2735@columbia.edu.
† Department of Biomedical Engineering, Columbia University.
‡ Current address: Department of Manufacturing Engineering and Engineering Management, City University of Hong Kong, Kowloon, Hong Kong.
§ Department of Electrical Engineering, Columbia University.
|| Mailman School of Public Health, Columbia University.

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of cells in 1 μL of blood), exclusion of cross-contamination in signal from monocytes (which also express CD4 surface marker), and delicate control of flow rate (especially in methods involving cell capture, as the binding of T-cells takes place optimally within a window of shear stress[12]).

In an alternative strategy to cell capture inside a microfluidic channel, cells can be captured on a track-etch membrane with 3 μm pores and CD4+ T-cells imaged by fluorescence imaging and counted using a computer algorithm for image analysis. A prototype for CD4 counting based on this method has been elegantly demonstrated,[13] with high correlation coefficients (0.91−0.95) to cell counts obtained by flow cytometry. Recently, Inverness Medical has also developed a POC CD4 counting device. For all reported strategies, miniaturization of the peripheral instrumentation and detection system to produce signals that can be collected rapidly and automatically reported (and without the need for complex or bulky optical or electrical systems) continues to present a challenge for their use in truly remote settings.

Previously, we had developed a POCKET immunoassay for detection of protein markers (such as HIV antibody) from human blood.[18] This technology used an amplification chemistry (based on silver reduction on gold nanoparticles) that is compatible with microfluidics and can be detected by simple optics. While it works well with detection of proteins, it is less appropriate for detection of cells: unlike small molecule analytes or proteins for which many copies can be captured, only a small number of cells cover the detection area. (For example, using the silver reduction strategy in the POCKET assay, <0.5% of the optical cross-sectional area would be blocking out the background light, in an example of 2000 CD4+ T-cells captured within the microfluidic chamber of this study.) In this study, we amplified the signal using chemiluminescence,[19,20] a highly sensitive detection method that also works well with continuous-flow microfluidics (i.e., as the signal, which is emitted light in this case, resides in the same location even as the signal development reagents passes over the capture zone, unlike ELISA where the optically active product would flow away from the capture zone). Despite these advantages, chemiluminescence has yet to be explored in detail for cell counting in microfluidics.

Our strategy uses passive capture of CD4+ T-cells followed by chemiluminescence detection. For cell capture, since only a small volume of blood (~10−20 μL) is obtained from a finger prick, we used two strategies to maximize the capture of a small number of CD4+ T-cells. First, building on previous methods for passive cell capture,[12,21] we designed a microfluidic chip with microfabricated pillars (covering 40% of the chamber area when viewed top-down) coated with anti-CD4 antibody. Compared to open channels, the pillars exhibited an increased surface area for contact with cells (Figure 1A). These monolithic structures can be easily mass manufactured using techniques such as injection-molding of plastic. Second, we designed the pillars according to shape and dimensions[22] (3 curved structures and a central post that trace out a 150 μm diameter circle, with inner exit flow-paths for cells) (Figure 1) to produce a range of shear stresses, in order to ensure that the target cells experience the optimal values of shear stress for binding to surfaces[12] in some regions of the chamber. Computational modeling of fluid flow around our chambers showed that different regions of the microfluidic chamber exhibited a range of 0.4−4 dyn/cm^2 in shear stress (with a flow rate of 2 μL/min), which encompasses the window of shear stresses (1−3 dyn/cm^2) that is optimal for CD4 cell capture as reported in other works.[12]

To test the effectiveness of capturing CD4+ T-cells, we passed a small volume (3 μL) of human whole blood through the chip and used fluorescence microscopy to count the number of T-cells in the chamber. Throughout this study, we used anti-CD3 antibody for detection in order to avoid signal from captured monocytes (which express CD4 but not CD3 on the cell surface[13]). For the initial studies of verifying cell capture, we used an anti-CD3 antibody conjugated with Alexa Fluor 488 and visualized the captured T-cells under a fluorescent microscope (Figure 1B). First, we noted that the densely arranged pillars, while facilitating capture of target cells, might also result in nonspecific binding of cells that do not express CD4. After testing a range of design parameters (spacings between traps from 25 to 50 μm and flow rate from 1 to 5 μL/min), we found that designs with small spacings resulted in high nonspecific binding, whereas in our current design, only 3% to 15% of immobilized CD3+ T-cells bound to the surface via nonspecific interactions (based on control experiments in which blood samples were introduced into chips without anti-CD4 antibody immobilized on the pillars; 329, 103, and 175 cells were nonspecifically captured when 3 μL of samples with CD4 counts of 982, 1093, and 1497 cells/μL were introduced, respectively). Therefore, the vast majority of CD3+/CD4+ T-cells that bound to the pillars did so specifically via interaction with the anti-CD4 antibody rather than nonspecific binding.

Next, we flowed human whole blood samples of different counts of CD4+ T-cells (as determined by flow cytometry) through our microfluidic chip. After subtracting from the measurements the count due to nonspecific binding, we plotted the number of captured cells as a function of CD4 count (Figure 2). We obtained a correlation coefficient between our assay and standard flow cytometry of 0.94. While we intended fluorescence to be used only for examining cell capture, and not in our final device (in fact, we noted difficulties in counting individual T-cells in clumps), we observed that for CD4 counts close to 200 cells/μL (the clinically significant cutoff), the numbers of captured cells exhibited CVs of 1.4−22%. When 250 cells/μL was set as a threshold for diagnosis of AIDS, the fluorescence-based method yielded sensitivity of 92% and specificity of 89%, with 95% confidence intervals of 16% and 21%, respectively.

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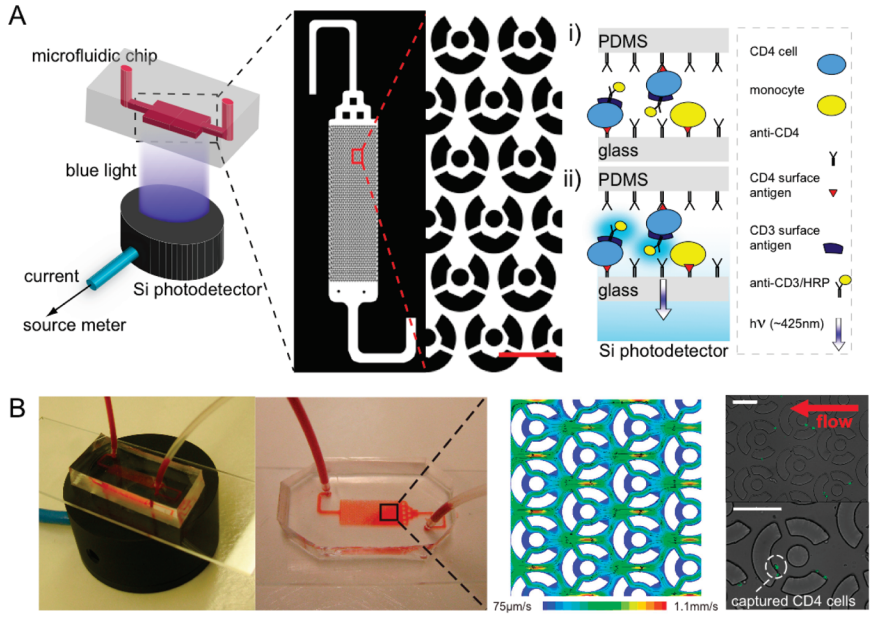

Figure 1. Schematic diagrams and pictures of a microfluidic chip for capturing CD4+ T lymphocytes using chemiluminescence-based detection. (A) Schematic diagram of setup (left), design of microfluidic traps (middle; scale bar is 100 μm), and schematic diagram of the cell-capture strategy (right). For cell capture, whole blood is introduced into the chip, followed by rinsing of unbound cells (including red blood cells) (top). As both monocytes and T lymphocytes are CD4+, these two cell types remain within the chip, however only the T lymphocytes have CD3 surface antigens and are hence labeled with CD3-IgG-HRP. Therefore, when luminol and hydrogen peroxide is introduced, the generation of light would only result from the presence of CD4 cells (bottom). This blue light is detected by a silicon photodetector and is then converted to a photocurrent. (B) Pictures of a microfluidic chip on the photodetector (left), microfluidic chip with human whole blood inside (middle left), computational modeling of fluid flow inside the cell-capture chamber (middle right), and fluorescence micrographs of T-cells captured in the traps (right). The right panel shows the microtraps at 20× magnification (top) and 40× magnification (bottom). The green dots correspond to captured CD4 cells labeled with anti-CD3-IgG conjugated to Alexa Fluor 488. The fluorescence images of the stained cells sometimes appear speckled and hence may not reflect the true size of the CD4 cell. Size of cells measured under phase contrast was around 9−11 μm. The red arrow indicates the direction of flow when the blood sample and buffers were introduced into the chip. The scale bars are 100 μm.

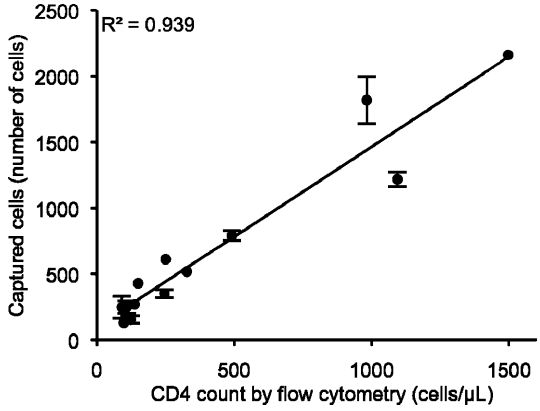

Figure 2. Quantitation of captured T-cells from 3 μL of human whole blood using a microfluidic chip (as performed by manual counting of fluorescently labeled T-cells), as a function of CD4 counts obtained by flow cytometry. We ran samples with 13 different CD4 counts (ranging from 91 to 1497 cells/μL) with up to three replicates per data point. The standard deviations are shown.

The capture efficiency, or percentage of CD4 T-cells available for capture (including cells trapped at inlets that did not even flow into the chamber) that were ultimately immobilized in the chip, was about 80%. Moreover, we observed that over 70% of captured T-cells reside in the low-flow rate areas near the traps. Hence, we concluded that the microfabricated pillars captured available T-cells with high efficiency, as intended by the microfluidic design.

Having verified the ability of the device to capture CD4+ T-cells, we tested the ability of the device to perform cell counting using chemiluminescence-based detection. We used a flow rate for chemiluminescent substrate (luminol and H_2O_2 mixture) of 5 μL/min (high flow rates led to shearing of captured cells, while low flow rates resulted in low signal). A silicon photodiode was used to monitor the magnitude of the light from the CL reaction as a function of time (Figure 3A). Before the luminol/H_2O_2 reached the capture chamber (~180 s), the measured current corresponded to the photodiode dark current. After ~300 s, the measured current corresponded to the photocurrent generated by the light from the CL reaction. We analyzed a time-averaged signal of the photocurrent over 200 s toward the end of a 8 min period. (Because of fluctuations in current, we found that the time-averaging of signal was critical: for example, CVs for raw current were very large, up to 82%, compared to 1−30% for time-averaged readings, depending on the amount of IgG-HRP used.) We obtained excellent correlation between the measured photocurrent and CD4 counts (Figure 3B), with a correlation coefficient of 0.99. The analysis of a total chemiluminescence signal emanating from many captured cells, rather than the optical signal from individual cells, could also compensate for any variations due to cell-to-cell variations in the expression of the CD3 marker.

We also explored the possibility of shifting the linear range of detection by using different concentrations of chemilumninescent substrates. We observed that the overall correlation coefficient, for samples with cell counts ranging from 0 to 1500 cells/μL, decreased when we changed concentration of IgG-HRP (during labeling of the T-cells) from 20 to either 10 μg/mL (due to

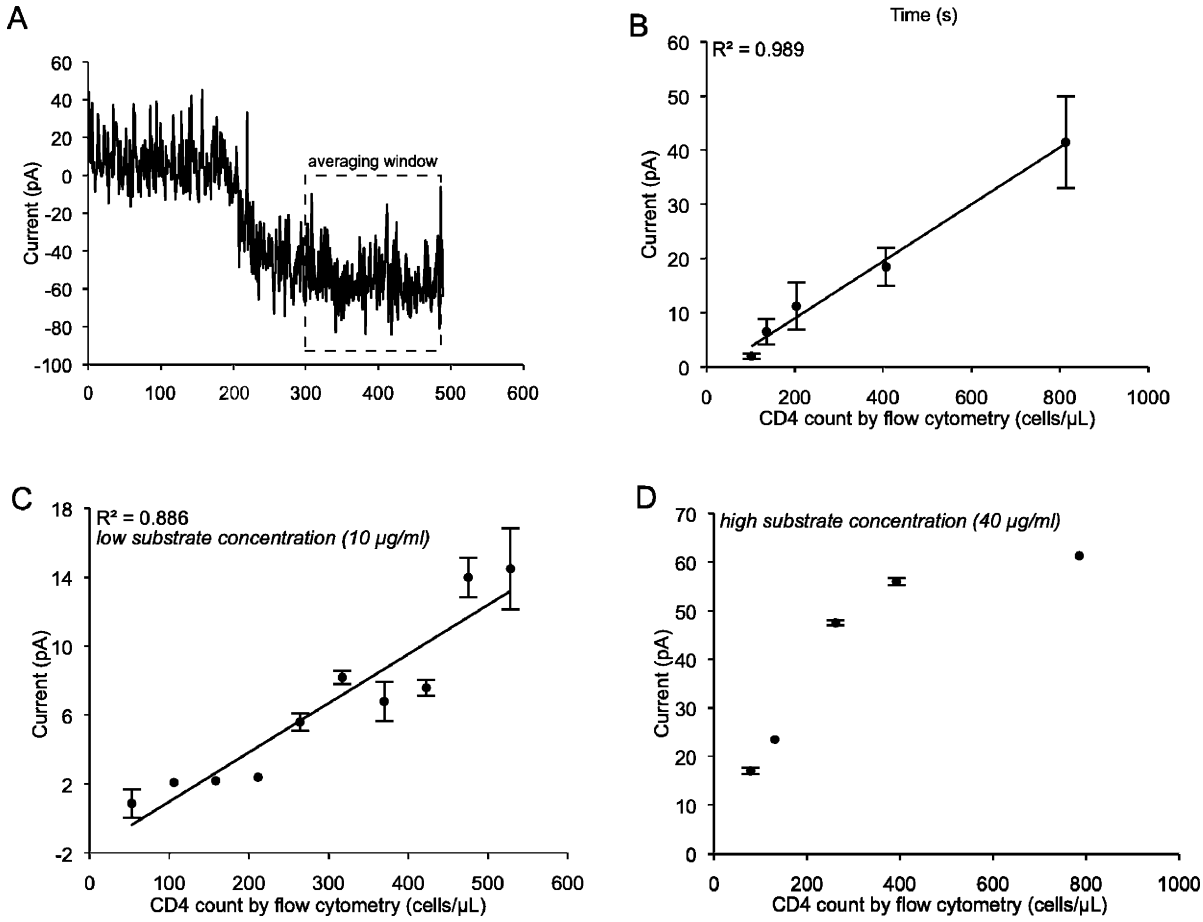

Figure 3. Detection of captured T-cells by chemiluminescence. (A) Plot of transient photocurrent observed during chemiluminescence-based detection. Following introduction of luminol and H_2O_2, photocurrent signals were collected for ~8 min. The photocurrent from 0 to 200 s was dark current and background noise, as little light was generated (while the substrate was reaching the capture chamber and the reaction was taking time to generate light). The photocurrent became negative upon detection of light. A time average of the last 200 s over the 8 min period was measured. (B) Plot of time-averaged photocurrent against CD4 counts obtained via flow cytometry. The concentration of IgG-HRP used during the blood staining process was varied from 20 μg/mL. There were two replicates per data point, and the standard deviations are shown. (C) Plot of time-averaged photocurrent against CD4 counts obtained via flow cytometry for two different concentrations of IgG-HRP. The concentration of IgG-HRP used during the blood staining process was varied from 20 to 10 μg/mL and 40 μg/mL. Up to two replicates were performed for each data point, with the standard deviations shown.

reduction in the signal-to-noise of the photocurrent) or 40 μg/mL (due to saturation in the amount of light as the oxidation reaction became increasingly substrate-limited) (Figure 3C). Nevertheless, since the photocurrent can reach up to several hundreds of picoamps, our system can enumerate CD4 cells even at low CD4 counts by increasing the concentration of IgG-HRP, when low ranges of CD4 counts are of special interest (e.g., in following AIDS progression in diagnosed patients).

Next, we tested the accuracy of our calibration curves for predicting the positive or negative statuses of the patient. With the use of the two graphs where good correlation was observed across all cell counts (i.e., at 20 and 10 μg/mL of IgG-HRP) and 11 and 4.8 pA as the thresholds for diagnosis of AIDS (corresponding to the clinically significant 200 cells/μL threshold), our device displayed a sensitivity of 90.9% with a 95% confidence interval of 10.8% and a specificity of 100%. (Shifting our photocurrent thresholds would trade sensitivity for specificity, depending on the clinical need.) Hence, we believe this device exhibits the desired analytical features of a semiquantitative POC diagnostic test that centers on the clinical thresholds around 200 cells/μL.

In the future, for true POC use, this method could be integrated with automated fluid handling methods,[23] for labeling the captured CD4 T-cells with the anti-CD3 detection antibody on the chip, as well as for washing and delivery of chemiluminescent substrate. This method could also be integrated into a miniaturized detection system; although the current system requires no external light source, it comprises a source meter which weighs only 5 lb but requires ground electricity. Another important point of focus will be to explore reagent-stabilizing approaches to minimize the effect of environmental variations (such as temperature) on the performance of the device.[24,25]

This study complements previous capture methods for CD4 counting, which were based primarily on counting of cells under fluorescence or optical microscopy, with other work based on

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electrical detection.[14,26] While passive cell capture has been used previously for POC CD4 counting,[12−15] we attempted to minimize the complexity of device, user training, and time to result by circumventing the needs for careful control of flow rate, cofabrication of electrodes, bulky equipment (including microscopes), or manual counting by a technician. In addition, compared with other imaging-based methods,[13,27] our assay can deliver favorable performances (with the correlation coefficient of our device being 0.99) without the need for complex imaging systems, which may be especially attractive for point-of-care applications in truly remote settings.

In summary, we have demonstrated the use of chemiluminescence, a detection method that is sensitive, compatible with continuous-flow microfluidics, and requires no external light source, in a microfluidic cell counting device using low volumes of human whole blood. The result can be provided automatically and digitally immediately after the end of the assay. Such a detection system may ultimately be integrated into rapid and low-cost devices for POC cell counting, such as CD4 counting for diagnosis of AIDS, in resource-limited settings.

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SUPPORTING INFORMATION AVAILABLE

Description of the materials and methods used. This material is available free of charge via the Internet at http://pubs.acs.org.

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