Microfluidic differential immunocapture biochip for specific leukocyte counting

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Enumerating specific cell types from whole blood can be very useful for research and diagnostic purposes—e.g., for counting of CD4 and CD8 T cells in HIV/AIDS diagnostics. We have developed a biosensor based on a differential immunocapture technology to enumerate specific cells in 30 min using 10 μl of blood. This paper provides a comprehensive stepwise protocol to replicate our biosensor for CD4 and CD8 cell counts. The biochip can also be adapted to enumerate other specific cell types such as somatic cells or cells from tissue or liquid biopsies. Capture of other specific cells requires immobilization of their corresponding antibodies within the capture chamber. Therefore, this protocol is useful for research into areas surrounding immunocapture-based biosensor development. The biosensor production requires 24 h, a one-time cell capture optimization takes 6–9 h, and the final cell counting experiment in a laboratory environment requires 30 min to complete.

INTRODUCTION

The counting of specific leukocytes finds many applications in disease diagnosis and management. One of the most common applications is enumerating CD4 and CD8 T cells for HIV/AIDS diagnostics. There are 34 million people infected with HIV/AIDS worldwide, with a lack of testing facilities in resource-limited settings[1–4]. An important diagnostic biomarker for HIV/AIDS is the absolute count of the CD4$^+$ and CD8$^+$ T lymphocytes in whole blood[5–8]. Current standard practice for CD4$^+$ and CD8$^+$ cell counting uses flow cytometers that are readily available in developed nations but that are not easily available in underdeveloped regions because of limited resources and lack of technical personnel to maintain and operate the instruments[9]. A flow cytometer is not suitable for point-of-care applications because of its high cost, which can range from $50,000 to over $125,000, with a maintenance cost of around $10,000 per annum[10]. There is clearly a need to have a portable, economical and robust device for HIV/AIDS diagnostics. Therefore, a lot of effort has been expended to develop such specific cell counters that would bring the AIDS and other diagnostics tests to the point-of-care settings in resource-limited regions of the world.

In a flow cytometer, specific cell enumeration is done after manual lysis of red blood cells (RBCs) from whole blood, with subsequent labeling of the desired cells with the conjugated antibodies. For example, the sample is labeled with CD4-, CD45- and CD3-conjugated fluorescent antibodies for optically based CD4 T cell counting[11]. However, cells can also be counted electrically by the Coulter principle[12]. To this end, the use of miniaturized Coulter counters on a chip for electrical cell counting has been previously reported[13–16]. In these devices, AC impedance analysis with microfabricated electrodes can be used to electrically interrogate and count the cells[17–20].

A microfluidic device can provide a solution to develop a point-of-care cell counter. Many microfluidic cell counting devices have been developed in the past—e.g., the microfluidic image cytometers that obtain CD4 counts by analyzing images of fluorescently labeled cells[21–23]. An integrated microfluidic system capable of counting the CD4$^+$ and CD8$^+$ T cells using fluorescently labeled antibodies with off-chip sample preparation has also been reported[7]. Most of these methods necessitate manual processing of the blood samples. Immunochromatographic strips that compare labeled CD4$^+$ leukocytes with a reference strip provide qualitative results without sufficient accuracy[24]. Impedance microcytometers that perform electrical interrogation of cells provide information about different cellular processes[16,17,25], differentiate between chemically infected cells[26] and also differentiate different cell types on the basis of size and membrane capacitance[20,23,27]. However, electrical cell counting is not sensitive enough to differentiate between cells with similar cell morphologies[28]—e.g., to differentiate between a CD4$^+$ and CD4$^-$ T lymphocyte.

Development and overview of the protocol

Human blood is composed of 45% of cells with five million erythrocytes, as compared with only 7,000 leukocytes in 1 μl of blood. Specific leukocytes such as CD4 T cells are of the order of 50–1,000 cells per μl (**Supplementary Table 1**). Electrical cell counting can differentiate cells based on size and membrane properties depending on the frequency of the interrogation signal. However, differentiating cells of the same morphology is a challenge; e.g., a CD4$^+$ T lymphocyte cannot be differentiated from CD4$^-$ lymphocytes just by electrical interrogation. In response to this challenge, we previously developed a microfluidic biosensor based on a differential immunocapture electrical cell counting technology to enumerate specific types of white blood cells from 10 μl of whole blood[29–32]. We have used this method to count CD4 and CD8 T cells for HIV/AIDS diagnostics with high accuracy[29].

The schematic of the differential immunocapture technique is shown in **Figure 1a** (ref. 29). The representative layout of the corresponding biochip is shown in **Figure 1b** (ref. 29). First, whole blood (10 μl) is infused into inlet '*a*', and the lysing reagent composed of saponin and formic acid is introduced into inlet '*b*' (**Fig. 1b**). This makes the surrounding medium for erythrocytes more hypotonic, and it results in the complete lysing of RBCs.

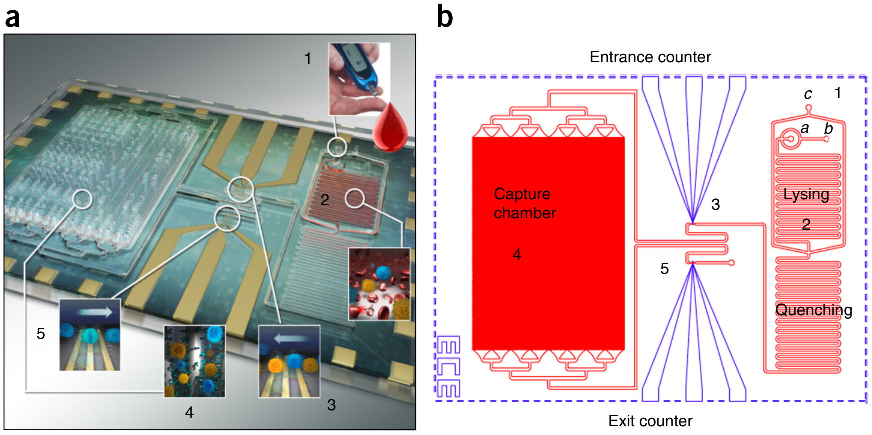


Figure 1 | The schematics of the differential immunocapture biochip[29]. (**a**,**b**) (1) Whole blood (10 µl), lysing and quenching solutions are infused into the biochip at *a*, *b* and *c* inlet ports. (2) Erythrocytes are lysed and the leukocytes are preserved using custom-made lysing and quenching buffers that are mixed with whole blood. (3) The leukocytes pass over co-planar platinum microfabricated electrodes and are counted. (4) Antibodies specific to particular cells (e.g., monoclonal CD4 T cell antibodies) are initially adsorbed on the capture chamber. CD4 T cells are captured as they interact with the antibodies in the chamber. (5) Remaining leukocytes get counted again with the second counter. The difference in the respective cell counts gives the concentration of the cells captured.

This lysing time must be optimized to completely lyse all RBCs. The quenching buffer composed of sodium carbonate and PBS is then infused into the inlet port '*c*' (**Fig. 1b**) to halt the lysing process and to preserve the osmolarity of the lysed sample. The remaining leukocytes pass through the microfabricated electrodes, which count the cells and provide the 'entrance counts' before cells pass into the capture chamber. CD4 T cell antibodies are immobilized in the capture chamber by adsorption surface chemistry. As the leukocytes pass through, $CD4^+$ T cells are captured and retained by the antibodies. We optimized the shear stress for maximum capture efficiency and minimum nonspecific capture. The remaining cells then pass through the second counter and provide an 'exit count'. The difference between the entrance and exit lymphocyte counts gives the number of captured CD4 T cells[29]. The fabricated biochip is shown in **Supplementary Figure 1**. The required steps to perform the protocol with their associated figures in the manuscript are given in **Table 1**.

We have compared our biochip counts with the control flow cytometer counts and have shown the correlation coefficient of $R^2 = 0.92$ for CD4 T cells and $R^2 = 0.92$ for CD8 T cell counts using HIV-infected blood samples[29]. Our biochip takes whole blood as input, thus eliminating the need for off-chip sample preparation and effectively reducing the assay time as well. In this protocol, we will provide a comprehensive stepwise procedure to replicate our biosensor for CD4 and CD8 T cell enumeration, which can be easily adapted for any other specific leukocyte-counting applications.

Experimental design

Development of the protocol should start from the electrical cell counting, as it is at the heart of the protocol. To optimize the lysing and quenching of erythrocytes and capture efficiency of desired cells, electrical cell counting needs to be performed.

Electrical cell counting. In our biochip, cells are counted electrically based on the Coulter counting principle by applying an external electrical field. At low frequencies (~300 kHz), the cell's membrane becomes nonconducting, and the change in the impedance is based only on the cell size[14]; thus, lymphocytes can be easily differentiated from granulocytes and monocytes. However, at high frequencies (~1–2 MHz), the cell membrane becomes more permeable and the impedance change depends on the cell membrane capacitance[14,19]. For example, monocytes have higher cell membrane capacitance and have extensive folding in the membrane as compared with neutrophils, so they can be differentiated. Thus, by probing the cells at these frequencies, we can differentiate between erythrocyte debris and different types of leukocytes; i.e., lymphocytes can be differentiated from granulocytes and monocytes.

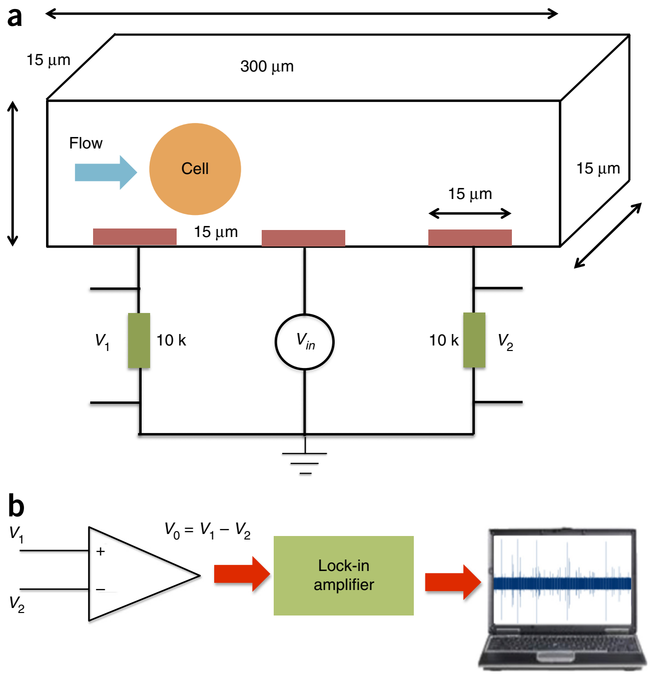
We have used microfabricated co-planar platinum electrodes for electrical cell counting. The width of the electrode and the spacing between the electrodes is 15 µm. The fabrication protocol involves electrode design for cell counting, lithography, metal evaporation and lift-off procedures (PROCEDURE Steps 1–17). The top and cross-sectional views with the specific dimensions of the counters are given in **Supplementary Figure 2**. The electrodes were aligned to a 15 × 15 µm fluidic channel[29]. For cell passage over the electrodes, a bipolar pulse is generated (**Supplementary Fig. 3**). The height of the pulses depends on the cell size, whereas its width depends on its speed. Thus, by selecting the appropriate threshold, the number of the pulses provides the count of the cells (**Supplementary Fig. 4**).

The electronic setup to generate the signal for cell counting as they pass over the electrodes is given in **Figure 2**. An input signal

TABLE 1 | Different processes of the protocol and their respective PROCEDURE steps and figures.

Process	Steps	Figures
Microelectrode fabrication	1–17	**Supplementary Figure 2**
Fluidic layer fabrication	18–36	**Fig. 4**
PDMS biochip fabrication	37–48	**Supplementary Figure 1**
Capture chamber immobilization		
• Antibody + BSA blocked	49–58	–
• BSA blocked	59–61	–
Capture efficiency optimization	62–80	**Fig. 5** **Supplementary Figures 4** and **5**
CD4/CD8 T cell counting experiment	81–98	**Figs. 7, 8** **Supplementary Figure 3**

Figure 2 | Electronic setup of the biochip. (**a**) A 15 × 15 µm cross section of the counting channel, with a cell flowing from left to right. Input signal, V_{in} 5 V at 303 kHz and 1.7 MHz, is given to the middle electrode of the biochip. The output voltages V_1 and V_2 are acquired across the 10 kΩ (10 k) resistors, respectively. Adapted with permission from ref. 31, Royal Society of Chemistry. (**b**) The output voltages are fed to the differential amplifier that cancels the common mode noise between V_1 and V_2. The output voltage of differential amplifier is fed to the lock-in amplifier. Data are collected using a DAQ card with sampling frequency of 500 kHz.



of 5 V at 303 kHz and 1.7 MHz is fed to the middle counting electrode. Relative impedance was measured using the Wheatstone bridge circuit, by acquiring output voltages V_1 and V_2 across the 10 kΩ resistors, respectively[30]. The output voltages are then fed to a differential amplifier that cancels the common mode noise in between V_1 and V_2. The output voltage of differential amplifier is fed to the lock-in amplifier to further remove any unwanted noise. Data are collected using a data acquisition (DAQ) card with a sampling frequency of 500 kHz (ref. 29).

Lysing/quenching optimization. The effective lysing and quenching of erythrocytes needs to be optimized to preserve the remaining leukocytes. The efficient erythrocyte lysis and quenching steps are extremely critical, as they will prevent the cell debris from clumping and clogging the biochip. Multifrequency interrogation of the cells is used to optimize the process. The lysed and quenched blood is interrogated at 303 and 1.7 MHz (ref. 29). **Figure 3** shows the scatter plot of the opacity (i.e., pulse amplitude at high frequency divided by the pulse amplitude at low frequency) versus the impedance at low frequency[29]. As shown in **Figure 3**, we can easily distinguish leukocytes from erythrocyte debris. The two cell distributions, i.e., lymphocytes and granulocytes + monocytes, are also clearly differentiated. Cells are not permeabilized from a lysing time of 3 s (**Fig. 3a**). However, as the lysing time is increased to 8 s, lymphocytes start to permeabilize and move toward the left to make a distinct distribution, as shown by the arrows (**Fig. 3b**)[29]. Thus, the blood:lysing and blood:quenching ratios were adjusted to optimize the lysing/quenching process. We found the optimized ratios as 1:12 and 1:6.3, respectively[29].

Specific cell capture efficiency optimization. The efficient and specific capture of cells requires the optimization of shear stress, capture chamber design and flow rate. In our earlier study, we tried to capture CD4 T cells in a planar capture chamber, but only 45% of the cells were captured[29]. We improved the design and included pillars, which resulted in increased surface area and more cell interactions with the antibodies. The optimized spacing between the pillars depends on the optimized shear stress for cell capture and the size of the desired cell to be captured. From our previous studies, we found that CD4 and CD8 T cell capture is most efficient at ~0.1 dyn/cm^2. For CD4 and CD8 T cell counts, which are a lymphocyte subpopulation (size 7–10 µm), we have selected an 11-µm spacing between the pillars. Another important consideration is that spacing should be approximately the same size as the cells to allow for a higher probability of cellular interactions with the pillar surface. The shear stress for four different designs with varying pillar spacings of 8, 11, 14 and 17 µm between the posts was explored via simulations (**Supplementary Fig. 5**), and the 11-µm post-spacing design was used experimentally, as it provided the optimal spacing with respect to the average cell diameter of 7–10 µm for lymphocytes[29] along with the necessary shear stress for optimal CD4 and CD8 cell capture. **Figure 4a** shows a scanning electron microscopy (SEM) image of the polydimethylsiloxane (PDMS) pillars in the chamber. The conceptual interaction of the cell with the antibody attached to the pillars is shown in **Figure 4b**. The footprint of the capture chamber is 2.5 cm × 1.4 cm × 100 µm. With 11-µm spacing and 40-µm diameter of pillars, there are ~134,560 pillars in the capture chamber[29].

Blood samples (10 µl) were lysed and quenched on the chip, and the highest capture efficiency was found by varying the capture chamber shear stresses. **Figure 5** shows an illustration of the capture efficiency optimization. As the first step, blood is lysed and the lysing is quenched on-chip. The lysed output is taken before entering the capture chamber and is labeled with anti-CD4, anti-CD5 and anti-CD3–conjugated antibodies. The incubated sample was run through a flow cytometer, and CD4 cells were counted in the sample (**Fig. 5a**). Second, to quantify nonspecific cell capture

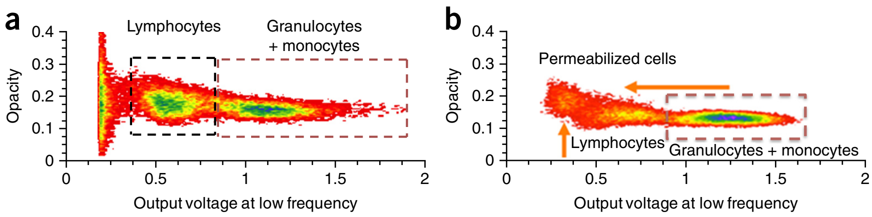


Figure 3 | Scatter plots of opacity versus pulse amplitude at low frequency[29]. (**a**) Cells are not permeabilized after a lysing time of 3 s. (**b**) As the lysing time is increased to 8 s, the cells start to permeabilize. Lymphocytes start to permeabilize and move toward the left to make a distinct distribution, as shown by the arrows. Adapted with permission from AAAS from Watkins *et al.*[29].

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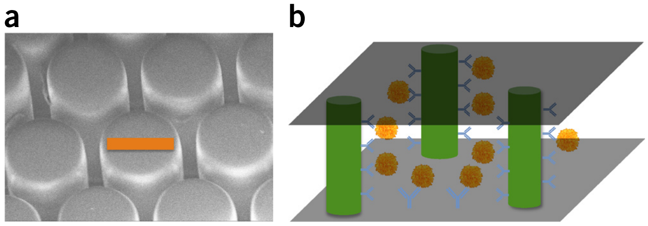

Figure 4 | Capture chamber for CD4$^+$ and CD8$^+$ T cell capture. (**a**) SEM image of the PDMS pillars in the capture chamber. Scale bar, 40 μm. (ref. 29). (**b**) Graphical illustration of the antibody and cell binding on the pillars[29]. Adapted with permission from AAAS from Watkins *et al.*[29].

(CD4– lymphocytes), blood was lysed and quenched on-chip, and then the cells were passed through a BSA-blocked capture chamber. The output is labeled with conjugated antibodies and run through a flow cytometer (**Fig. 5b**). Third, specific CD4 T cell capture is found by passing the cells through an antibody + BSA-blocked capture chamber. The output is labeled with conjugated antibodies and run through a flow cytometer (**Fig. 5c**). The flow cytometry analysis of the labeled cells is shown in **Supplementary Figure 6**. The side scatter versus CD45 plot shows a distinct population of lymphocytes, which can be gated out (**Supplementary Fig. 6a**). The gated lymphocyte population is plotted in a CD3 versus CD4 fluorescent plot. The top right quadrant gives the CD4$^+$ T lymphocytes (**Supplementary Fig. 6b**). Similarly, the bottom right gives CD4$^+$ monocytes. In our earlier study, we optimized the capture efficiency procedure and had shown the maximum capture efficiency for CD4$^+$ and CD8$^+$ T cells to be 98.28 and 90.14%, respectively[29], obtained at 0.11 dyn/cm^2. The capture efficiency decreases at shear stresses larger than 0.11 dyn/cm^2, probably because the CD4$^+$ or CD8$^+$ T cells did not have enough interaction time with the antibodies on the surface of the pillars in the capture chamber. Absolute counts of specific CD4 T cells can be obtained by taking the difference of lymphocyte counts before and after capture using two electrical counters and multiplying it with the cell capture ratio. Cell capture ratio is defined as the CD4$^+$ lymphocyte/CD4$^-$ lymphocyte capture.

Steps 62–80 in the protocol can be used for the CD4 and CD8 T cell capture optimization. However, for point-of-care application purposes, cell capture needs to be optimized just once, and then the counter can be used in low-resource settings without the need for a flow cytometer. However, Steps 62–80 should be repeated again for any other specific cell-counting application.

Applications of the protocol

One of the primary applications of the differential immunocapture assay that we produced was selective enumeration of CD4$^+$ and CD8$^+$ T cells for HIV/AIDS diagnostics. We performed our earlier study with healthy samples (n = 18) and HIV-infected blood samples (n = 32) and enumerated CD4$^+$ and CD8$^+$ T cell counts using our protocol[29]. These results were compared with flow cytometry control cell counts, which were obtained from the Carle Foundation Hospital Laboratory. We have shown a correlation coefficient of $R^2 = 0.89$ for CD4$^+$ T cells and $R^2 = 0.937$ for CD8$^+$ T cells for healthy samples[29]. For HIV-infected samples, we found $R^2 = 0.9201$ for CD4$^+$ T cells and $R^2 = 0.9197$ for CD8$^+$ T cells[29]. The high correlation with the gold standard (i.e., flow cytometry) suggests that our differential immunocapture technology can provide specific cell enumeration for point-of-care applications, especially in resource-limited regions of the world.

Graduate students and professional researchers may find this protocol very useful for their research in areas such as microfabrication and biosensor development. Our biochip, along with its electronic sensors and electrodes, can be used to count other particle types and other types of cells (in addition to blood cells)—e.g., somatic cells, cells from tissue or liquid biopsies, urine, other fluids and so on. The same protocol can also be used to capture other specific cell types used for disease diagnostics: for example, it can be used in childhood cancer diagnostics by specifically enumerating CD19 and CD33 cells in the capture chamber. Our approach to capture specific types of leukocytes is broadly applicable, for example, to profiling the immune system for different cell types, and capturing those cells for genomic and proteomic analysis can be very useful for many applications in research. The specific immunocapture can be easily adapted for other specific cell counting applications. For the capture of different cell types, their specific antibodies need to be immobilized in the chamber. Upon considering the different surface expression of the antigens, the required shear stress for maximum capture efficiency will be different and will need to be optimized again (Steps 62–80 can be used for this purpose). Antibodies can also be dry-spotted before bonding electrodes to the fluidics layer in order to use the integrated biochip (**Supplementary Fig. 1**).

Limitations of the protocol

Our protocol presents a limitation on the concentration of the target cell to be captured. The minimum detectable cell concentration depends on the specific cell capture efficiency and

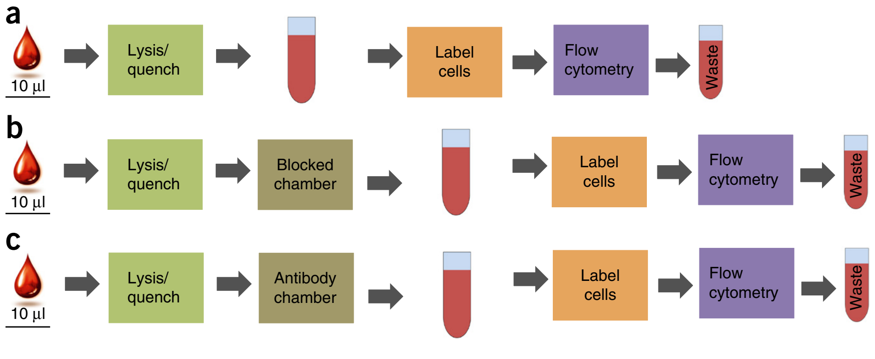

Figure 5 | Illustration of capture efficiency optimization[29]. (**a**) CD4 cells before capture. 10 µl of blood is lysed and quenched on the chip. The lysed output is labeled with conjugated antibodies and run through a flow cytometer to count CD4 cells in the sample. (**b**) Nonspecific cell capture; 10 µl of blood is lysed and quenched on the chip. The cells are passed through the BSA-blocked capture chamber. The output is labeled with conjugated antibodies and run through a flow cytometer. Nonspecific capture of CD4$^+$ and CD4$^-$ lymphocytes is found. (**c**) Specific CD4$^+$ T-cell capture; 10 µl of blood is lysed and quenched on the chip. The cells are passed through the antibody + BSA-blocked capture chamber. The output is labeled with conjugated antibodies and run through a flow cytometer.

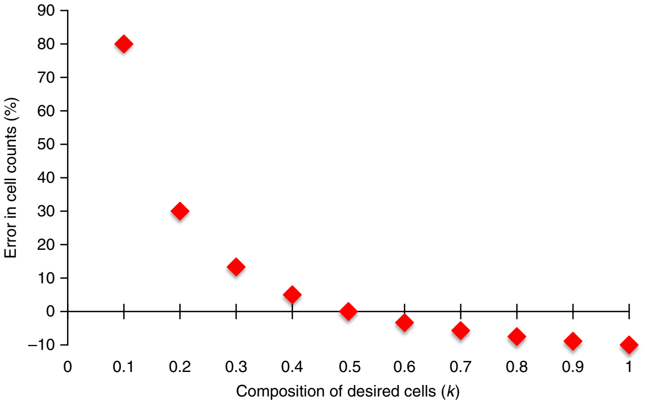

Figure 6 | Percentage of error in specific cell counts. Shown are varying percentage levels of the desired cell population in the total cell population with 90% specific cell capture and 10% nonspecific cell capture. *k* is the percentage of specific cells comprising the total cell population.

nonspecific cell adsorption in the chamber. The following error analysis is used to find the limit of the target cell concentration found in the differential cell counter approach. Let N be a distinct cell population obtained from the entrance counter. Let $N = A + B$, where A and B are the two subpopulations that comprise the total N cell population. Let k be the percentage of N comprising A cells such that $A = kN$. Then, B will be $B = (1-k)N$. We wish to capture cells of type A specifically, with a capture efficiency of x%. The total captured cells in the capture chamber will be given as follows:

$$\text{Captured cells} = xA + yB = kxN + (1-k)yN = (kx - ky + y)N$$

Percentage of error in the A cell count can be calculated by the following equation:

$$\text{Error}(\%) = \frac{A-(kx-ky+y)N}{A} = \frac{k-kx+ky-y}{k}$$

The percentage of error to count specific cell A with varying levels of the A cell population in the total cell population k, with 90% specific A cell capture and 10% nonspecific B cell capture, is shown in **Figure 6**. The A cell concentration should be >30% of the total N population—i.e., $k > 0.3$—to get <10% error in cell counts. However, for a reduced nonspecific cell capture, the minimum detectable concentration can be increased.

In our previous study to enumerate CD4 and CD8 T cells for HIV/AIDS diagnostics, we experimentally showed CD4 T cell counts of as low as 40 cells per µl (ref. 29). We used 32 HIV-infected blood samples and obtained an average percentage of error of 5.3% for CD4 T cell counts and 7.4% for CD8 T cell counts when compared with the standard flow cytometer counts provided by Carle Foundation Hospital[29]. Furthermore, for repeatability studies, we obtained an ~5% coefficient of variance[29].

One of the operating limitations of our biosensor is clogging of the counting channel, which can be prevented by appropriate selection of the counting channel aperture dimensions. The aperture of our counting channel is 15 × 15 µm, and it is selected to prevent clogging, as all the leukocytes are smaller than 15 µm in size. However, for other cell types or bigger particles, the counting channel cross-section should be increased to prevent any clogging. The counting channel can also get clogged by RBC debris clumps. We have used saponin in our lysing reagent, which is known to dissociate the RBC clumps. If RBC clumps clog the counting channel, the concentration of the saponin should be incrementally increased by 0.03% (vol/vol) in the lysing reagent. In our experiments, the current saponin concentration was sufficient to dissociate the debris clumps.

Another practical limitation is the clogging of the immunocapture area—i.e., the capture chamber—because of high concentrations of captured cells. The current capture chamber dimensions will allow ~200,000–400,000 cells to be captured. Currently, our capture chamber is good enough to capture CD4 and CD8 T cells from >100 µl of blood, whereas our protocol requires only 10 µl.

MATERIALS

REAGENTS

- CD4-unconjugated antibody (Beckman Coulter, clone 13B8.2, cat. no. IM0398)
- CD8-unconjugated antibody (Thermo Fisher Scientific, clone 3B5, cat. no. MHCD0800)
- CD4-conjugated antibody (Thermo Fisher Scientific, clone S3.5, cat. no. MHCD0420)
- CD8-conjugated antibody (Affymetrix eBioscience, clone OKT8, cat. no. 53-0086-42)
- CD45-conjugated antibody (Affymetrix eBioscience, clone HI30, cat. no. 25-0459-42)
- CD3-conjugated antibody (Affymetrix eBioscience, clone OKT3, cat. no. 45-0037-42) ▲ **CRITICAL** We have used the above antibodies in our experiments. Using antibodies with different clones might produce variable results. However, antibodies with the same clones can be used from other suppliers too.
- PBS, 1× (Thermo Fisher Scientific, cat. no. 10010-023)
- PBS, 10× (Thermo Fisher Scientific, cat. no. AM9624)
- Acetone, deionized (DI) water, isopropanol (Laboratory-supplied chemicals)
- Formic acid (Sigma-Aldrich, F0507)
- Sodium carbonate (Sigma-Aldrich, S7795)
- BSA (Sigma-Aldrich, A7030)
- Saponin (Sigma-Aldrich, 47036)
- LOR 3A photoresist (MicroChem, cat. no. G516606)
- Remover PG (MicroChem, cat. no. G050200)
- S1805 photoresist (MicroChem, cat. no. 10018321)
- SU8-10 photoresist (MicroChem, cat. no. Y131259)
- SU8-100 photoresist (MicroChem, cat. no. Y131273)
- SU8 developer (MicroChem, cat. no. Y020100)
- CD26 developer (Shipley, cat. no. 38490)
- 3-Mercaptopropyltrimethoxysilane (Gelest, SIM6476.0-100GM) ! **CAUTION** 3-Mercaptopropyltrimethoxysilane is carcinogenic. A face shield and double gloves are required along with standard personal protective equipment (PPE) while working with this reagent. It must only be used under the fume hood.
- Fresh blood samples drawn into an EDTA-coated Vacutainer tube (BD, cat. no. 366643) and analyzed within 12 h of the draw ! **CAUTION** Handling of any blood sample should be done by considering all blood samples as potentially infectious. Work should be carried out in a Biosafety Level 2 (BSL-2) facility, as directed by your institution's Division of Research Safety (DRS). PPE includes protective gloves, a lab coat and a face shield while working with blood samples. All experiments with human blood should follow DRS guidelines and regulations. ! **CAUTION** Blood collection from human subjects should be done through proper Institutional Regulatory Board Approval, and informed consent should be obtained from donors.

EQUIPMENT

- NanoFlow metering system pump (Eksigent) ▲ **CRITICAL** The Eksigent NanoFlow metering pump has four individual channels and an HPLC feedback system that helps in maintaining the constant flow rates with much less flow-rate variation.
- Lock-in amplifier (Zurich Instruments, HF2LI)
- Differential amplifier (Zurich Instruments, HF2CA)
- DAQ card (National Instruments, NI PCIe-6361, cat. no. 781050-01)
- DAQ card cable (National Instruments, SHC68-68-EPM, cat. no. 192061-02)
- Injection valve, two-position, six port (Rheodyne Systems, MHP 7980-500-1)
- Syringe, 1 ml (Fisher Scientific, cat. no. 22-253-260)
- EDTA Vacutainer tubes (Becton Dickinson and Company, cat. no. 366643)
- Centrifuge tubes, 2 ml (Fisher Scientific, cat. no. 05-408-138)
- Guava easyCyte plus flow cytometer (EMD Millipore)
- Conical tubes, 50 ml (Corning, cat. no. 430897)
- Vortex mixer (Cole-Parmer, cat. no. EW-04726-01)
- Timer (Cole-Parmer, cat. no. EW-94440-00)
- Serological pipette tips, 10, 200 and 1,000 µl (Fisher Scientific)
- Platinum evaporation pellets (Kurt J. Lesker Company)
- Glass wafers (University Wafer, ID. 516)
- Si wafers, 4 inch (single-side primed, Silicon Quest International)
- Glass Petri dishes (Corning, 70165-152)
- Microscope slides (Fisher Scientific, cat. no. 12-550B)
- Microfluidic components (IDEX Health & Science)
- Microbore polytetrafluoroethylene (PTFE) tubing (Cole-Parmer, EW-06417-11)
- Ultramicrobore PTFE tubing (Cole-Parmer, EW-06417-74)
- PDMS, Sylgard 184 silicone elastomer kit (Dow Corning, Ellsworth Adhesives, cat. no. 184 Sil Elast kit, 0.5 kg)
- Hot plate (Thermo Scientific, HP131220-33Q)
- Adhesive tape (Ultron Systems, cat. no. 1007R-4.0)
- Conductive silver epoxy (MG Chemicals, 8331S-15G)
- Harvard PHD Ultra pump (Harvard Apparatus, 703007)

Software

- ZI Control (to operate lock-in amplifier: https://www.zhinst.com/products/hf2li)
- Eksigent control software (to control Eksigent pump for fluids pumping to biochip. Software provided with the system)
- LabVIEW (to control the switching valve and data acquisition from the biochip: http://www.ni.com/download-labview/)
- MATLAB (to analyze the collected data: https://www.mathworks.com/downloads/)
- FCS Express flow cytometry (to analyze the collected data from flow cytometer: https://www.denovosoftware.com/site/downloadresearch.shtml)

REAGENT SETUP

Quenching reagent Add 2.301 g of sodium carbonate and 84.7 ml of 10× PBS to a bottle filled with 315.3 ml of DI water. This gives the following concentrations: 21.1785% (vol/vol) 10× PBS and 2.301 g of sodium carbonate. Mix it vigorously and wait for 15 min until all bubbles are removed before use. ▲ **CRITICAL** The quenching reagent can be stored for 3–6 months at room temperature (i.e., 25 °C) or at 4 °C in a refrigerator. However, if it is stored at 4 °C, it should always be brought to room temperature before use.

Lysing reagent Add 200 mg of saponin to 480 µl of formic acid into a bottle filled with 399.5 ml of DI water. This gives the following concentrations: 0.12% (vol/vol) formic acid and 0.05% (wt/vol) saponin[29]. Mix vigorously and wait for 15 min until all bubbles are removed before use. ▲ **CRITICAL** The quenching reagent can be stored for 3–6 months at room temperature or at 4 °C in a refrigerator. However, if it is stored at 4 °C, it should always be brought to room temperature before use.

Blocking solution (PBS + 1% (wt/vol) BSA) Add 50 ml of 1× PBS to a 50-ml conical tube. Weigh 500 mg of BSA and add it to PBS. Mix at high speed using a vortex mixer until all particles are dissolved. Wait for 15 min until all bubbles are removed before use. ▲ **CRITICAL** The quenching reagent can be stored for 3–6 months at room temperature or in a 4 °C refrigerator. However, if it is stored at 4 °C, it should always be brought to room temperature before use.

PROCEDURE

Microelectrode fabrication ● TIMING 6 h

▲ **CRITICAL** Steps 3–16 must be performed in a clean room.

1| Draw the microelectrode device design using the AutoCAD software (the specific design used for our biosensor is available as **Supplementary Data**: 'Biochip Design_NP.dwg').

2| Submit the mask design to a vendor for mask making (parameters: 5 inch, Dark Field Right Read Down) and printing (parameters: Front Range PhotoMask).

3| Clean the 4-inch Pyrex wafer with acetone, isopropyl alcohol and DI water (in order), and then dry it using nitrogen gas.

4| Perform the dehydration bake at 110 °C for 5 min with a subsequent 5-min cool-off at room temperature.
▲ **CRITICAL STEP** After baking, the subsequent cooling at room temperature is important to bring down the wafer temperature before spinning the photoresist.

5| Spin LOR 3A photoresist on the wafer using the following settings: 0–500 r.p.m. in 1 s (acceleration setting) with a 2-s hold time, and then at 500–3,000 r.p.m. in 2 s (acceleration setting) with a 35-s hold time.
▲ **CRITICAL STEP** Place enough LOR 3A to cover the entire wafer before spinning; this helps greatly with chip yield.

6| Perform the soft bake for 5 min at 183 °C, with a follow-up of 5-min cool-off at room temperature.
▲ **CRITICAL STEP** Cool-off time is necessary to prevent any 'wrinkle' formation on the film.

7| Spin S1805 photoresist (place enough S1805 to cover the entire wafer) on the wafer at the following spin settings: 0–500 r.p.m. in 1 s (acceleration setting) with a 5-s hold time, and then at 500–4,000 r.p.m. in 3 s (acceleration setting) with a 40-s hold time.

8| Soft-bake the wafer again for 90 s at 110 °C with a follow-up of 5-min cool-off at room temperature.
▲ **CRITICAL STEP** This step is important to prevent the wafer from sticking to the mask during exposure.

9| Place the mask and the wafer in the mask aligner and expose them for 3.7 s (required exposure energy: 28 mJ/cm^2). This is calculated by dividing the exposure energy by a mask aligner's lamp power output (units of mW/cm^2).

10| Perform the post-exposure bake for 60 s at 110 °C.

11| Develop the wafer in CD-26 developer for 20 s.
▲ **CRITICAL STEP** Simply let the wafer sit in the developer (no agitation, swirling and so on; this can cause features to break off of the wafer).
? TROUBLESHOOTING

12| Rinse the wafer in DI water for 2 min (by simply swirling the wafer gently in a static dish of DI water).

13| Examine the wafer under the microscope. Make sure that there is no underdevelopment or overdevelopment of the photoresist.
■ **PAUSE POINT** It is recommended that the wafer be processed immediately for plasma etching and metal deposition. However, if needed, it can be stored at room temperature in the dark for up to a week.

14| Place the wafer in the plasma etcher system for 20 s at 50% power before placing it in the evaporator for metal deposition.
▲ **CRITICAL STEP** Make sure that the pattern side of the wafers is facing up.

15| Place the wafers in the metal evaporator and deposit 250 Å of titanium and then 750 Å of platinum per wafer.

16| After evaporation, place the wafers (upside down) in a lift-off processing box filled with Remover PG, which has been already warmed to 70 °C on a hot plate; leave the box for 10 min. Refill the processing box with fresh Remover PG and place it back onto a 70 °C hot plate for another 20 min.
! CAUTION Be sure to dump liquid into a nonhalogenated solvent waste container.
▲ **CRITICAL STEP** You can leave the wafers in Remover PG for longer to be sure of a clean lift-off. If residual metal pieces are left on the wafer, you can use cleanroom swabs with Remover PG or acetone to wipe them away. Be careful not to be too abrasive with wiping, as electrodes can be ripped apart even with a clean-room wipe. You do not have to be so careful if you are wiping in areas on the wafer that do not have electrodes.

17| Dice the wafers with a dicing saw to get individual electrode pairs.

Double-layer microfluidic design fabrication ● TIMING 2 h

▲ **CRITICAL** Steps 20–36 must be performed in a clean room.
18| Draw the microfluidic design of the biochip using the AutoCAD software (the specific design used for our biosensor is available as **Supplementary Data**: 'Biochip Design_NP.dwg').

19| Submit the mask designs to a vendor for masking (parameters: 5 inch, Dark Field Right Read Down) and printing (parameters: Front Range PhotoMask).

20| Clean the 4-inch silicon wafer with acetone, isopropyl alcohol and DI water (in order), and then dry it with nitrogen gas.

21| Perform a dehydration bake at 110 °C for a minimum of 5 min with a follow-up of 5-min cool-off at room temperature.

22| Dispense 4.5 ml of negative photoresist SU8-10 at the center of the wafer, making sure to avoid bubbles.

23| Spin the negative photoresist SU8-10 on the wafer for a thickness of 17.5 μm (first layer) at the following settings: 0–500 r.p.m. in 5 s (acceleration setting) with a hold for 5 s, and then at 500–1,750 r.p.m. in 4 s (acceleration setting) with a hold for 30 s. The final deceleration time is 3 s.

24| Pre-soft-bake the wafer at 65 °C for 2 min.

25| Soft-bake the wafer for 5 min at 95 °C with a follow-up of 10-min cool-off at room temperature.

26| Place the mask and the wafer in the mask aligner and expose them for 9.5 s. The exposure energy for 17.5 µm is 200 mJ/cm^2. Exposure time is calculated by dividing the exposure energy by a mask aligner's lamp power output (units of mW/cm^2).

27| Perform postexposure bake for 1 min at 65 °C, and then for 2 min at 95 °C with a follow-up of 5-min cool-off at room temperature. Hide the alignment marks with a paper strip.

28| Spin the negative photoresist SU8-100 on the wafer for a thickness of 100 µm (second layer) at the following settings: 0–500 r.p.m. in 5 s (acceleration setting) with a hold for 5 s and at 500–3,000 r.p.m. in 8.3 s (acceleration setting) with a hold for 30 s. The final deceleration time is 3 s.

29| Soft-bake the wafer for 10 min at 65 °C and then for 30 min at 95 °C with a follow-up of 10-min cool-off at room temperature. Remove the cover on the alignment marks.

30| Place the mask and the wafer in the mask aligner, and perform the alignment before exposure.

31| Expose them for 21.4 s. The exposure energy is 450 mJ/cm^2.

32| Perform post-exposure bake for 1 min at 65 °C, and then for 10 min at 95 °C with a follow-up of 5-min cool-off time. Blow N_2 at the back of the wafer for extra cooling-off of the wafer.

33| Develop the wafer in a SU-8 developer for 12 min (development time is found from data sheet of SU8-100 photoresist, and it relates to the thickness of the pattern exposed). Slowly shake the wafer for the whole time.

34| Rinse it with isopropyl alcohol and then dry the wafer with N_2.

35| Hard-bake the wafer at 110 °C for 10 min with a follow-up cool-off time of 5 min at room temperature.

36| Place the silicon wafer into a glass Petri dish and secure the edges with clean-room tape.
■ **PAUSE POINT** This wafer can be reused, and it can be kept in the clean room for extended periods.

PDMS device fabrication ● TIMING 2 h

37| Place the open bottle of 3-mercaptopropyltrimethoxysilane to silanize the Si mold surface to prevent sticking of the PDMS to the mold.

38| Place both the Si mold and the silane in the desiccator, and let them sit under vacuum for 60 min.
! CAUTION 3-Mercaptopropyltrimethoxysilane is carcinogenic. The above Steps 37 and 38 should be done under a fume hood, with a face shield and double gloves along with standard PPE.

39| Weigh the PDMS elastomer and the curing agent at a ratio of 1:10. For one Si mold, we used 2 g of curing agent and 18 g of elastomer. Mix them well and pour the resulting solution onto the mold.
▲ **CRITICAL STEP** The thickness of the PDMS should not exceed 1 mm in height from the surface of the silicon wafer.

40| Place the mold with PDMS in the desiccator.
! CAUTION Slowly allow air into the desiccator while opening it (may cause damage otherwise).

41| Let it sit for 20 min. Next, remove the mold from the desiccator and remove the bubbles with a nitrogen gas gun (repeat this step two or three times, until no bubbles appear).

42| Place the mold into the oven or on a hot plate for baking to harden. It can be baked at 65 °C overnight. For urgent experiments, it can also be baked at 110 °C for 20 min.
▲ **CRITICAL STEP** Ensure that the wafer is left on a level surface for the entire duration of the curing process.
■ **PAUSE POINT** The cured uncut PDMS can be kept for longer periods of time at room temperature.

43| Remove the mold from the oven and cut out the PDMS with a knife or a razor. Each device can be cut to three individual modules: i.e., lysing, counter and capture chamber (**Supplementary Fig. 7**).
? TROUBLESHOOTING

44| Place the cut PDMS piece under a microscope and drill inlet and outlet holes at the designated spots for microfluidic connections.
? TROUBLESHOOTING

45| Wash the PDMS devices in the solvent hood with acetone and then with isopropyl alcohol, and finally dry them with N_2. Clean the microelectrode chips with the same process.

46| Treat the PDMS device and the glass electrodes with oxygen plasma for 60 s at 50% power.

47| Take out the devices and bond them together.

48| For proper alignment of electrodes with the PDMS microfluidic counter module, use the mask aligner.

Capture chamber immobilization (antibody + BSA blocked) ● TIMING 2 h

▲ CRITICAL As mentioned in the Experimental design section, Steps 62–80 can be used for CD4 and CD8 T cell capture optimization. This requires two separate capture chambers to be prepared via Steps 37–48. One of these is then loaded with antibodies and blocking solution (Steps 49–58), and the second one is loaded only with blocking solution (Steps 59–61). These are both used in later steps to analyze the nonspecific capture of cells in the capture chamber during optimization. Both of these chambers can be serially prepared and kept at room temperature for 1–3 h for further experiments.

49| Pipette out 90 µl of 1× PBS into a centrifuge tube.

50| Add 10 µl of CD4-specific antibody solution into the centrifuge tube.

51| Mix the solution well with a pipette—i.e., move it up and down approximately five times—or vortex the sample gently for a few seconds.

52| Keep the pipette at 100 µl and take out all of the antibody solution into the pipette tip. Place the tip onto the 1-ml syringe and dispense the solution from the tip into the syringe.

53| Take a 1-ml syringe and fill it with 1× PBS. Connect the microfluidic connector to the capture chamber inlet (**Supplementary Fig. 7**) and flow PBS through it to remove all the air bubbles.
? TROUBLESHOOTING

54| Connect the antibody syringe to the microfluidic inlet connector that goes to one of the prepared capture chambers.

55| Use the Harvard PHD Ultra pump to flow the antibody solution through the capture chamber. Infuse 40 µl at a rate of 15 µl/min, and then incubate for 30 min at room temperature. Next, again infuse 40 µl at a rate of 15 µl/min, and then incubate for 30 min at room temperature.

56| Take 500 µl of 1× PBS into the 1-ml syringe. Connect it to the capture chamber. Flow ~100 µl of PBS at a rate of 10 µl/min to remove all the unadsorbed antibodies from the capture chamber.

57| Take 500 µl of the blocking solution—i.e., 1× PBS + 1% (wt/vol) BSA—into the 1-ml syringe. Connect it to the capture chamber. Flow 100 µl of blocking solution at a rate of 20 µl/min to block the capture chamber for nonspecific adsorption.

58| Incubate for 30 min at room temperature. The capture chamber can be kept at room temperature for 1–3 h for experiments.

Capture chamber immobilization (BSA blocked) ● TIMING 1 h

59| Prepare the BSA-blocked capture chamber. Take a 1-ml syringe and fill it with 1× PBS. Connect it to the microfluidic connector to the second capture chamber inlet, and flow PBS through it to remove all the air bubbles.

60| Take 500 µl of the blocking solution into the 1-ml syringe. Connect it to the second capture chamber. Flow ~100 µl of blocking solution at a rate of 20 µl/min to block the capture chamber for nonspecific adsorption.

61| Incubate for 30 min at room temperature. The capture chambers can be kept at room temperature for 1–3 h for experiments.

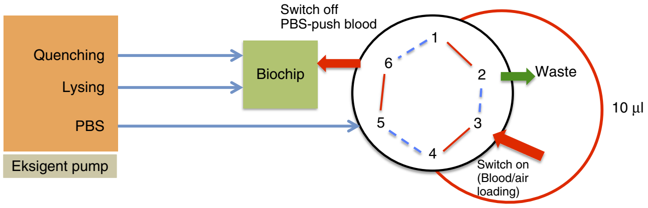

Figure 7 | Reagent loading mechanism into the biochip. Blood is metered off-chip using a two-port, six-position valve. Lysing, quenching and push buffer (1× PBS) reagents are loaded into the biochip using an Eksigent HPLC pump.

Capture efficiency optimization ● TIMING 6–9 h

▲ **CRITICAL** The cell capture efficiency optimization protocol is a one-time optimization. Once optimized, Steps 62–80 are not needed for recurring counting of specific cells—e.g., CD4 and CD8 T cells.

62| Pipette out 100 µl of blood from the Vacutainer tube, and then place the pipette tip into the 1-ml syringe, and dispense the blood from the tip into the syringe.

63| Infuse the blood, using the syringe in the holding coil, until a drop reaches the waste container (**Fig. 7**).
? TROUBLESHOOTING

64| Now using a 1-ml empty syringe, push a small air bubble into the holding coil.

65| Start infusing 1× PBS from a 1-ml syringe into the holding coil; it will start pushing the blood into the chip. As the blood reaches the start (10 µl mark) of the measurement tube, select 'START' on the LabVIEW file (**Supplementary Data**: 'Counter_Differential_NP.vi'), which will switch the valve connections. Next, the push buffer will start to push the blood at a rate of 1 µl/min to the blood inlet port on the biochip (**Fig. 7**).

66| Use the Eksigent HPLC pump to flow the lysing, quenching and push buffers (1× PBS) at flow rates of 12, 6.3 and 1 µl/min, respectively, at the designated ports in the cell-lysing module of the biochip (**Supplementary Fig. 7**).

67| Collect the lysed output from the outlet of the cell-lysing module without cell capture into a centrifuge tube (**Supplementary Fig. 7**).

68| Add 5 µl of CD4-, CD45- and CD3-conjugated antibodies to the tube.
! CAUTION Perform this step in the dark to prevent any photobleaching of the fluorophores.

69| Let the sample incubate for 15 min at room temperature in the dark.

70| Mix the sample using a pipettor or vortex it gently before running it through a flow cytometer.

71| Run the sample through a flow cytometer and acquire forward scatter, back scatter, green fluorescence and red fluorescence signals.

72| Use FCS Express software and plot the side scatter versus CD45 fluorescent scatter plot; the lymphocyte population can be gated as shown in **Supplementary Figure 6a**.

73| Plot the CD3 versus CD4 scatter plot, as shown in **Supplementary Figure 6b**. The top right quadrant gives the CD4 lymphocytes. Similarly, the bottom right gives the CD4 monocytes.

74| Count the total number of lymphocytes and CD4 T cells (**Supplementary Fig. 6**).

75| Now connect the BSA-blocked capture chamber (from Step 61) to the output of the cell-lysing module using ultramicrobore tubing.

76| Repeat Steps 62–74. The difference of the lymphocyte count from Step 74 (no capture) from the lymphocyte count (after BSA blocked chamber) will provide information on the nonspecific capture of cells.

77| Now connect the inlet of the antibody and BSA-blocked capture chamber (from Step 58) to the outlet of the cell-lysing module (**Supplementary Fig. 7**).

78| Repeat Steps 62–74. The difference of the lymphocyte count from Step 74 (no capture) from the lymphocyte count (after antibody + BSA-blocked chamber) will indicate the specificity of the CD4 T cell capture.

79| Vary the flow rates to optimize for the maximum capture of specific cells, such as CD4$^+$ T cells and CD8$^+$ T cells, and minimum capture of nonspecific cells.

80| By using Steps 72, 74 and 76, select the desired flow rate.
▲ **CRITICAL STEP** Approximately 5 μl of CD4, 5 μl of CD14-conjugated antibodies and 1 μl of DAPI can be mixed with 200 μl of 1× PBS solution and allowed to flow through the chamber to stain the cells. This can be used to image the capture chamber for the specific captured cells. Cells can also be fixed by flowing 100 μl of 4% (wt/vol) paraformaldehyde with 300 μl of 1× PBS through the chamber.
■ **PAUSE POINT** If the chamber remains hydrated, it can be preserved for many weeks for later analysis.

Running a CD4$^+$ and CD8$^+$ T cell counting experiment ● TIMING 30 min

81| The inlet of the antibody + BSA-blocked capture chamber is connected to the first counter. The outlet of the capture chamber is connected to the second electrical counter (**Supplementary Fig. 7**).

82| Turn on the lock-in amplifier instrument, load the ZI Control file for the lock-in amplifier (**Supplementary Data**: ‘lockin.zicfg’), and the LabVIEW data acquisition files (**Supplementary Data**: ‘Counter_Differential_NP.vi’).

83| Repeat Steps 62–66. The voltage pulses will start appearing for both counters and data will start recording. The amplitude histogram of the recorded data will look like that shown in **Figure 8a**. The differential of the lymphocyte count, the cell recovery after lysing and the nonspecific lymphocyte capture will give the absolute concentration of the CD4 T cells.
? TROUBLESHOOTING

84| Repeat the same experiment three times to find the coefficient of variance in the experiment.

85| *Running the LabVIEW file*. Install LabVIEW 2013 SP (or higher version).

86| Load the ‘Counter_Differential_NP.vi’ file (**Supplementary Data**).

87| On the front panel, give the file locations for the low-frequency and high-frequency data sets that are to be saved on the computer. Do this for both entrance and exit counters.

88| Click the run arrow under the menu bar.

89| Turn on the blood valve.

90| Load the blood sample.

91| Turn off the blood valve. The PBS will start pushing the blood from the 10-μl blood tube to the biochip.

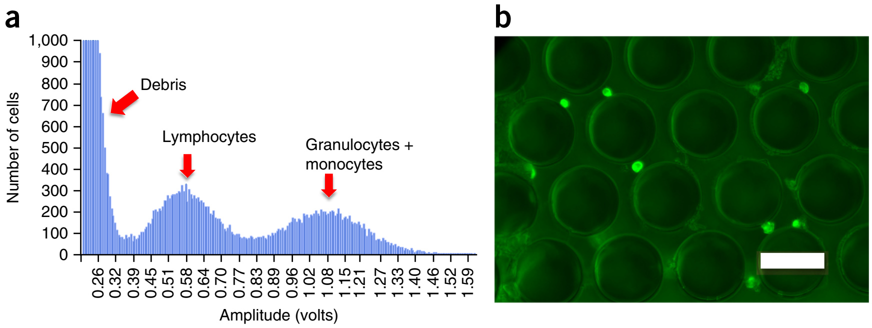

Figure 8 | Anticipated results[29]. (**a**) As the lysed cells pass through the electrical counter, voltage bipolar pulses are generated. The histogram of the peak amplitudes of the pulses provides information on the size distribution of cells. The histogram shows two distinct populations of leukocytes—i.e., lymphocytes and granulocytes + monocytes[29–32]. (**b**) False-colored image of the fluorescently labeled CD4 T cells captured between the pillars. Scale bar, 40 μm (ref. 29).

92| Click the 'record file' button on the file to start recording the data at a sampling rate of 250 kHz. The green round LEDs on the front panel will turn on.

93| *Running the lock-in amplifier operating software file*. Install Zi Control 15.01.

94| Run the ZI Control application software.

95| Open the 'Save' tab.

96| Load the settings file (**Supplementary Data**: 'lockin.zicfg') under the 'Save/load HF2 settings' panel.

97| Give the required output voltages and their frequencies to be generated under the 'Lock-in MF' tab.

98| The 'Auxiliary I/O' tab gives information about the demodulator connections to auxiliary output channels going to the DAQ card.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
11	Electrode features break	Swirling or agitation during development	Avoid agitation and swirling during development
43	PDMS sticks to the mold	Si mold surface is not properly salinized	Perform salinization for a longer time
44	Leaks in the PDMS devices	Hole punching in Step 44 caused ruptures	Use a new hole puncher
53	Trapped air bubbles	Insufficient wetting of the capture chamber	Flow ethanol before flowing 1× PBS through the chamber
63	Blood is not flowing through the holding coil	Valve or PTFE tubing is clogged	Replace the tubing and unclog the valve using 10% (vol/vol) bleach and then PBS
83	Pulses do not appear or data does not record	Incorrect selection of the analog output (AO) channels or faulty electrodes	Select the correct AO channels on the DAQ card or use the new electrodes

● TIMING

Steps 1–17, microelectrode fabrication: 6 h
Steps 18–36, microfluidic (double layer) fabrication: 2 h
Steps 37–48, PDMS device fabrication: 2 h
Steps 49–58, capture chamber immobilization (antibody + BSA blocked): 2 h
Steps 59–61, capture chamber immobilization (BSA blocked): 1 h
Steps 62–80, capture efficiency optimization: 6–9 h
Steps 81–98, CD4 and CD8 T cell counting experiment: 30 min

ANTICIPATED RESULTS

While replicating our protocol, we recommend replicating our electrical counter first. As the lysed cells pass through the electrical counter, it will generate the voltage bipolar pulses. **Figure 8a** shows the representative histogram of the peak amplitudes of the pulses, which provides information about the size distribution of cells. The histogram shows two distinct populations of leukocytes—i.e., lymphocytes and granulocytes + monocytes. With optimized shear stress, the desired cells will be captured as they flow through the capture chamber by their respective antibodies. **Figure 8b** shows a false-colored image of the fluorescently labeled CD4 T cells captured between the pillars.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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