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Microfluidic-based detection platform for on-the-flow analyte characterization

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ABSTRACT

While commercial flow cytometers are sophisticated analytical instruments extensively used in research and clinical laboratories, they do not meet the challenging practical requirements for point-of-care (POC) diagnostics. In this paper we will describe and illustrate a new detection technique that will enable a compact, microfluidic-based flow cytometer that satisfies POC specifications for performance, robustness, compactness, cost, reagent consumption, and ease of use. The technology has been demonstrated with CD4 counts in whole blood.

Keywords: *flow cytometer*, *opto-fluidics*, *on-the-flow analyte characterization*, *micro-fluidics*

1. INTRODUCTION

Flow cytometry is the process of measuring chemical and or physical characteristics of biological cells as they flow through an instrument in a fluid stream [1]. Virtually all modern commercial instruments rely on optical interaction with the bio-particles for characterization, through fluorescence, scattering, or absorption processes. And all use the same basic optical configuration, namely, intense illumination of the bio-particle as it speeds through a highly localized spot, which generally involves a complex arrangement of optics (e.g., lenses, mirrors, apertures, and filters). This highly focused beam of light is required to achieve usable sensitivity since the signal is proportional to the photon flux density. One of the major milestones in the evolution of flow cytometers was the introduction of lasers for the illumination [2] because of their inherently high flux density and small solid angle of emission, which can also simplify the optics. In this paper we introduce a fundamentally new design of the optical detection system that delivers high effective sensitivity (i.e., high signal to noise) without complex optics or bulky, expensive light sources to enable a flow cytometer that combines high performance, robustness, compactness, low cost, and ease of use.

Flow cytometers are indispensable tools in clinical diagnostics, such as in diagnosing cancer, AIDS, and infectious diseases during outbreaks, and also in microbiology and other areas. Chemical and or physical information is obtained about a moving object such as a biological cell, a virus, a molecule, or a sub-molecular complex, as it is transported in a fluid stream. The cost and size of existing flow cytometers preclude their use in resource limited settings, field clinics, water monitoring, agriculture/veterinary diagnostics, and rapidly deployable bio-threat detection.

A number of commercially available flow cytometers use multiple excitation sources, each focused on a well-defined location or region separate from the others. Light emitted from each source's region is typically analyzed with a series of beam splitters, filters, and photomultiplier tubes (PMTs) in order to detect and distinguish differently stained cells or cells that concurrently carry multiple dyes. Cells are typically stained in solution with different dyes prior to insertion into a cytometer, and the measurement event occurs as the cells traverse a detection region within a fluidic channel, at a speed of up to several meters per second. In the detection region, focused laser light (typically with an elliptical focus of 80 μm x 40 μm) excites the dyes on the cells. The resulting fluorescent light can be collected by a microscope lens, sorted by band pass filters, and detected by PMTs or avalanche photodiodes (APDs). For each spot excitation, a respective set of filters and detectors is needed, which is costly and leads to bulky instruments with critical requirements to maintain optical alignment. Since the detection region is small and the objects traverse it rapidly (typical dwell times are around 10 μsec), such flow cytometers have serious signal-to-noise ratio (SNR) limitations for weakly fluorescing cells. These limitations become more acute if multiple targets must be characterized and distinguished for counting or sorting.

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A major cost in flow cytometry applied in clinical diagnostics is the cost of reagents (e.g., antibodies and conjugated dyes). There are two ways to reduce the amount of consumables: First, one can reduce the required amount of analyte, for example, by employing microfluidic techniques; and second, one can reduce the amount of consumable per analyte volume. Reducing either of these quantities would, however, reduce fluorescent intensity. We propose to overcome this constraint by introducing a cost-effective and reliable technique to detect and distinguish weakly emitting particles.

2. SPATIALLY MODULATED FLUORESCENCE DETECTION

The technique that enables a compact micro-fluidic-based flow cytometer we term "spatially modulated fluorescence" detection [3]. It involves relative movement between an illuminated bio-particle and a selectively patterned environment. This produces a time-modulated signal that is analyzed with real-time correlation techniques. Correlating the detected signal with the known pattern distinguishes the fluorescence signal from the background noise to achieve improved signal-to-noise discrimination, and it provides precise information about particle speed and position. The improved S/N discrimination can be used to replace complex optics, fragile PMT detectors and bulky expensive light sources. The robustness and miniaturization arise from replacing expensive components with inexpensive ones that can be readily integrated on a fluidic chip and by eliminating the need for sophisticated optics and critical optical alignment.

2.1 Basic concept

The spatially modulated fluorescence detection technique enables an improved signal-to-noise (S/N) ratio for all types of measurements where light (fluorescent or scattered) originating from a moving particle in a channel is characterized. In conventional flow cytometry, the size of the excitation area is restricted approximately to the size of the particle. Our method uses a much larger excitation area to increase the total flux of fluorescence light that originates from a particle. Despite the large excitation area, the mask pattern enables spatial resolution in the micron range. This allows for independently detecting and characterizing particles with a distance (in flow direction) that can approach the dimension of individual particles. In addition, the concept is intrinsically tolerant to background fluorescence originating from fluorescent components in solution, fluorescing components of the chamber and contaminants.

Figure 2 displays the principle of spatially modulated fluorescence emission for particles moving through a fluid channel. For the sake of simplicity the example shown is for the case of a single color, but it can be extended to multiple colors. A large excitation area is used and a spatially patterned mask modulates the fluorescent light on its way to the detector. The time dependence of the signal is defined by the spatial structure of the stripes of the mask and the speed of the particle. In the following we refer to a mask which alternates between full transmission and no transmissions of the fluorescent light as a "binary mask". The recorded signal is analyzed by correlation techniques, and the intensity and time when the particle traverses the detection zone are accurately calculated. With state-of-the-art real-time correlation techniques, particle characterization up to a speed of a few m/s is possible.

In general a large variety of different mask patterns can be used, which includes periodic, chirp, and (pseudo-)random masks. Periodic masks have the advantage that the particle speed can be easily determined (e.g., with a Fourier transformation or electronically with lock-in techniques), however, there are some issues if the absolute position of the particle has to be determined very precisely or if multiple particles are in the detection area. These challenges can be efficiently addressed by using masks with a "randomly" defined on/off pattern. Real-time correlation of the measured time-varying particle signal from a random template can detect multiple particles in the detection area and determine their separation as well as their absolute position very precisely. In addition, the advantages of periodic and random masks can be combined by superimposing mask patterns; in this case the time-dependent signal includes both easily accessible speed and accurate particle position.

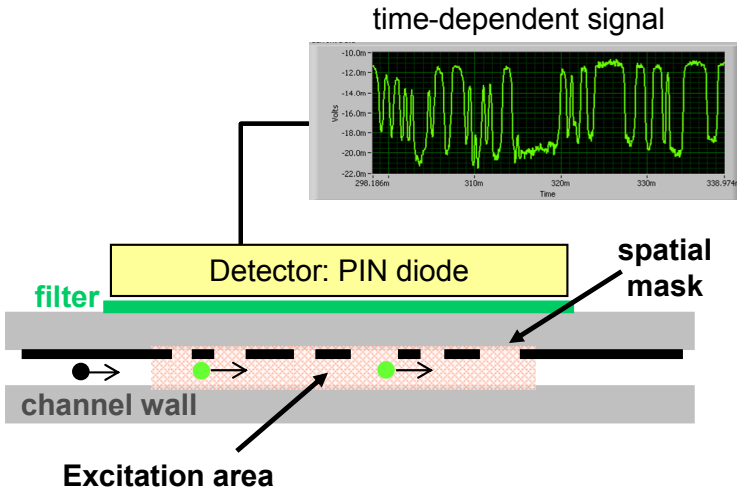

Figure 1: *Schematic cross-sectional diagram of a fluidic channel illustrating the concept of spatially modulated emission. A spatially patterned mask modulates the fluorescent light propagating toward the detector. The excitation light is blocked by a filter in front of the detector. Correlation techniques are applied to determine the position of the particle in real time.*

2.2 Set-up, lab demonstration and capabilities

The spatially modulated fluorescence technique was first demonstrated with a bench-top setup. Figure 2(a) schematically depicts the fluidic chip and the arrangement for fluidic handling, optical excitation, and collection of the spatially modulated fluorescence. The fluidic chip as shown in figure 2(b) is formed with two closely spaced quartz slides. The flow channel is typically 200 μm wide and 25 μm deep and is defined by a laser machined layer of double sticky transparent tape between the two 0.5 mm quartz slides. A pseudo-random mask pattern was photo-lithographically defined in a metal film deposited on the inside surface of the top slide. A representative pseudo-random mask pattern (1 mm along the flow direction, 100 μm high, minimum feature size 20 μm) is shown in Fig. 2(c). A syringe pump is used to control both the flow of the particle-containing (analyte) solution and the sheath flow. While sheath fluid is not required with our techniques, it can be used to optimize light-analyte interaction. We typically used a sheath-flow-to-analyte-flow ratio of 3:1:3 so that the width of the analyte flow was about 30 μm. The excitation light from a green (532 nm) laser is anti-resonantly coupled through the facet of one of the slides [4]. After several reflections within the slides and the fluid, a fairly homogeneous light intensity within the fluidic channel is achieved along the propagation direction, which coincides with the direction of the analyte flow. The excitation light was typically confined to a width of typically 50 μm right in the center of the 200 microns wide channel. The 50 μm wide excitation stripe was aligned to completely overlap the analyte flow. The excitation power density in the 25 μm x 50 μm cross section of the channel along the detection area was estimated by comparing the measured fluorescence intensity in the case of anti-resonant waveguide excitation with the intensity obtained by direct excitation. For most experiment the excitation power density was between 10 and 50 W/cm^2.

To illustrate the spatial modulation technique, Figure 3 shows a screenshot of the real-time data acquisition and correlation software which operates the system. The top of the screenshot in Figure 3 shows the design of the spatial mask, where the white areas represent the openings and the black areas the bars in a binary mask. The minimum feature size of the mask is defined as the width of the smallest openings or bars and determines the spatial resolution of the detector. The red signal is the measured signal for a weakly fluorescing polystyrene micro-bead (2μm diameter) as it traverses the patterned zone. Cross-correlation is used to characterize the degree of similarity between the recorded fluorescence signal S(t) and the ideal signal P(t) expected for a given mask pattern.

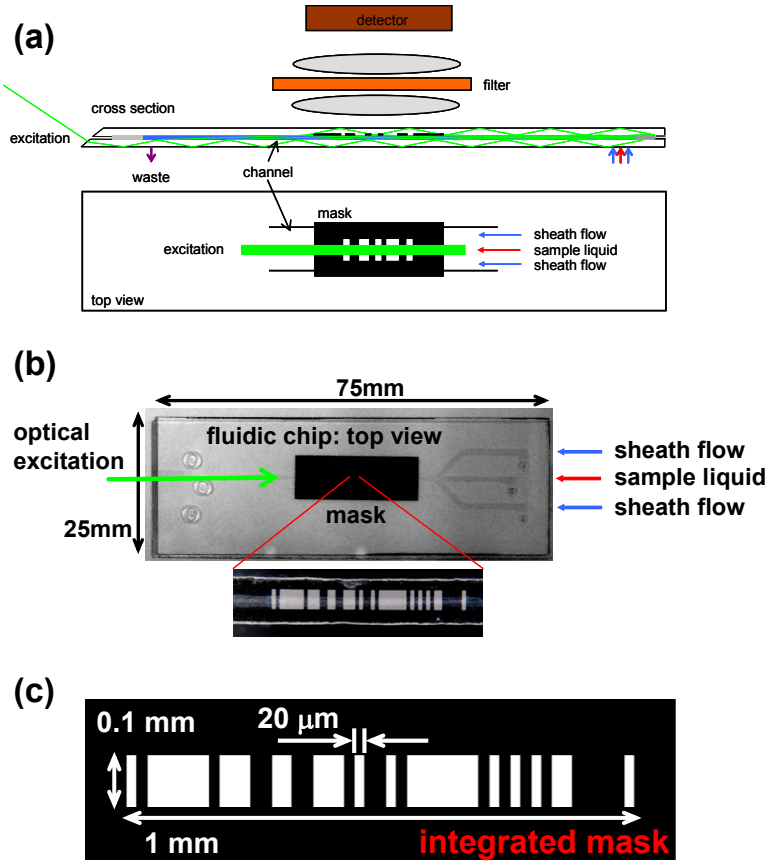

Figure 2: Experimental setup for spatially modulated collection of optically stimulated fluorescence emission from particles flowing through a fluidic chip: (a) schematic diagram, (b) fluidic chip with expanded image of integrated mask, and (c) typical mask dimensions used for CD 4 counting.

The correlation signal C(t) at time t is

$$C(t) \equiv \int_{-T/2}^{T/2} P(\tau)\, S(\tau + t)\, d\tau\,, \qquad (1)$$

where T is the transit time. The correlation signal consists of a broad, triangularly shaped curve, of duration 2T, topped by a sharp peak, as shown in the middle (white) graph of Fig. 3. The peak arises when the recorded signal perfectly aligns with the ideal signal; it is the signature for particle detection. The derivative of the correlation signal (green curve at the bottom in Fig. 3) provides quantitation for the particle. The particle position is accurately obtained by identifying the zero-point crossing between the pair of adjacent peaks with maximum positive and negative amplitudes. This accurately locates the position of the sharp peak in the correlation signal and determines the position of the particle at any time during its transit. The peak-to-peak height of the adjacent peaks is proportional to the integrated fluorescence intensity of the particle as it traverses the patterned zone. An absolute calibration of the signal can be obtained with calibration beads. To compare particles traveling at different speeds, the relative signals can be normalized to the transit times.

For the example in Fig. 3 the raw data display S/N ~ 1 while after the correlation analysis the exact particle position is reliably determined with S/N ~8. Alternative signal filtering techniques, for example, a low pass filter, could also improve S/N. However, the exact particle position within the excitation zone would be lost; this information is encoded in the high-frequency components of the spatial modulation signal. In addition, conventional filtering would not distinguish between particle signal and background.

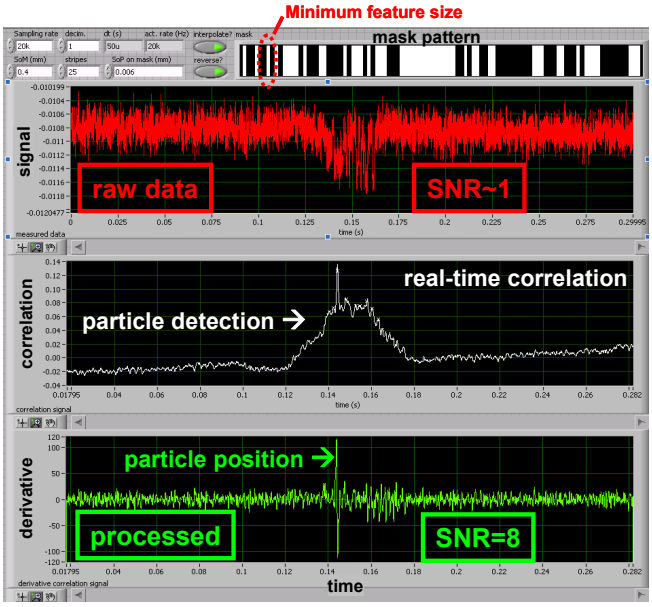

Figure 3: Screenshot of the real-time data acquisition and correlation software which operates the system. The measurement shows results for weakly fluorescing beads traversing the mask (minimum feature size 4 μm) at a speed of 10 mm/s. The excitation power in this example was ~ 5 W/cm^2

With our lab experiments we have successfully demonstrated the following capabilities of the spatially modulated detection technique:

- CD4 count (specific type of white blood cells) in whole blood for monitoring the immune status of HIV infected persons: this example demonstrates the intrinsic tolerance of the approach with respect to a large background signal from background fluorescence and undesired scattering by other particles (in this case red blood cells). These results are described more detailed in the next section.
- Tagged CD4-cells (dye: PE, emission maximum at 575 nm) were detected at flow speeds up to 0.5 m/s (excitation: 532 nm), with reliable detection extendable to flow speeds of a few m/s.
- Detection of individual cells (yeast, e-coli, BT) was demonstrated via native fluorescence (excitation 266 nm, fluorescence 340 nm) in combination with pattern recognition. Utilizing native fluorescence eliminates the complicated process of specific binding to tag target organisms with fluorescent dyes. This capability can be used to distinguish between inorganic particles and organic particles, e.g., cells.
- Individual tagged beads (diameter 6 μm) down to a particle separation of 20μm were successfully detected separately and enumerated. This demonstrates that multiple particles can be distinguished simultaneously within the detection area, which is particularly of interest if high count rates are required for high particle concentrations.
- Fluorescent beads with a diameter of 0.6 μm were detected at an excitation power density below 5 W/cm^2. Excitation power in this range can even be realized with conventional LEDs. Surface emitting LEDs with emission areas up to 0.8x0.8 mm^2 and power densities of more than 20 W/cm^2 are commercially available in the green and blue spectral range, and if higher power densities are required high-power edge-emitting LEDs or superluminescence diodes could be used.
- Particles were detected at a signal-to-noise ratio below 1. For illustration a measurement with a signal to noise ratio of about 1 is shown in Fig 2. Even in this case the particle can be clearly detected in the correlation signal and even the exact particle position can be reliably determined (S/R ~8).

The sensitivity and dynamic range of the spatial modulation technique are illustrated in Fig. 4(a) with a histogram of particle count as a function of fluorescence intensity for a mixture of fluorescent microbeads with three different diameters. For this experiment the excitation was provided by a 532 nm laser directly through the mask so that both the excitation and fluorescence of the beads were spatially modulated by the mask. The excitation power density was estimated to be 10 W/cm^2. The fluorescence and scattered excitation light were collected and collimated with a 20× microscope objective (NA=0.4), filtered with a 585 nm band pass filter with a spectral width of 40 nm, refocused with a 20× objective, and detected with a pixelated avalanche photodiode. The fluidic chip utilized both sheath flow and analyte flow, as shown in Fig. 2(b), in the flow ratio (sheath:analyte:sheath) of 5:1:5, with an analyte flow of ~10 μl/min. In the vertical dimension of the fluid channel the flow profile was parabolic, with the speed of the beads ranging from ~300 to 700 mm/s as shown in Fig. 4(b). All of the beads were tagged with the same dye with peak emission at 612 nm so that the emission intensity varied with bead diameter. The three clearly-separated peaks in Fig. 4(a) correspond from left to right to bead diameters of 0.6 μm, 2 μm, and 6 μm, respectively, with an intensity variation of ±30%. The absolute calibration in units of molecules of equivalent PE (MEPE) [5] was obtained by using a commercial flow cytometer (BD FACS) to compare the fluorescence intensity of the 2 μm beads with calibration beads of known intensity (BD Quantibrite). The results demonstrate a dynamic range of over three orders of magnitude. From the S/N performance we estimate that the detection limit is approximately 1000 fluorescence molecules for the current set-up, with the intensity from the 0.6 μm beads half an order of magnitude above this limit. Finally, as a point of reference, the intensity from CD4$^+$ lymphocytes stained with 1:1 conjugates of CD4-PE has been reported to correspond to ~5×10^4 MEPE [6]. This value is in excellent agreement with our measurements of fluorescence intensity from individual tagged CD4$^+$ lymphocytes as shown below.

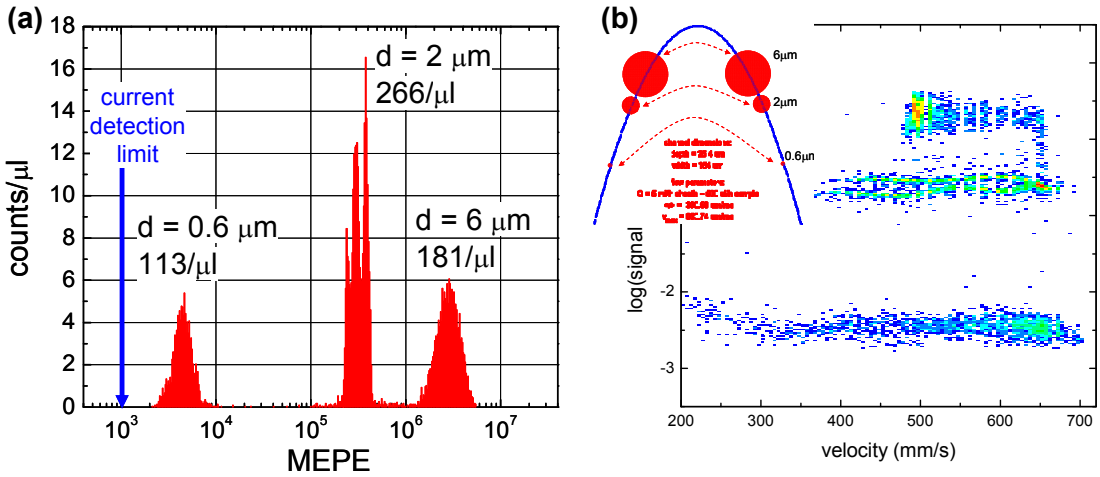

Figure 4: (a) Histogram of particle count as a function of fluorescence intensity for a mixture of fluorescent beads with three diameters d. The absolute calibration in units of molecules of equivalent PE (MEPE) was estimated by comparing the 2 μm beads with calibration beads. The detection limit with the current experimental set-up is ~10^3 MEPE

(b) Fluorescent intensity as a function of particle speed for the measurement shown in Fig. 4(a). Compared to the 0.6μm particle, the larger micro beads show a narrower speed distribution as flow focusing constrains them to a narrower range in the flow profile (as indicated in the inset).

3. CD4 COUNT IN WHOLE BLOOD

As a compelling demonstration of the utility of our spatially modulated detection technique, we performed measurements of absolute CD4+ and percentage CD4 counts in human blood, which are required for screening, initiation of treatment, and monitoring of HIV-infected patients. To benchmark our technique we performed a direct one-to-one comparison (not shown in this paper) of measurements on the same samples with a commercial instrument (BD FACSCount) and obtained excellent agreement for both absolute CD4 and percentage CD4. In this paper we will concentrate on early measurements of the concentration of CD4 T-lymphocytes marked with R-phycoerythrin (PE) in PBS buffer-solution. For the CD4 measurements we adjusted analyte and sheath flow so that the resulting average flow speed in the channel was 65 mm/s. From the Navier-Stokes equation [7] the maximum speed in the center of the channel was ~110 mm/s.

We applied a common, basic lab protocol to tag the CD4 cells. The blood samples were incubated with PE-conjugated CD4 antibodies for 20 or 40 min at 37 °C and diluted with PBS buffer solution for dilution factors in the range from 50 to 1.4. No lysing of the red blood cells or washing steps to separate the white blood cells was used. The fluorescence from the CD4 cells was collected through the mask by a 20x microscope objective (NA=0.4) and focused onto a pixelated APD detector.

We have tested a large variety of blood samples with different dilutions, blood/reagent ratio, incubation times, and temperatures. In the following we discuss the results for blood a blood sample diluted by a factor of 50.4 (sample A) as detailed in Table 1.

Table 1: Details on sample preparation for the CD4 test

Sample	whole blood (μl)	PE dye and antibody (μl)	PBS (μl)	dilution factor
A	20	8	1000	50.4

Figure 5(a) shows a histogram of detected CD4 cells as a function of fluorescent intensity for sample A. The plot exhibits two peaks, which are attributed to CD4 lymphocytes (right peak) and CD4 monocytes (left peak). The relative count rate of lymphocytes and monocytes and, more importantly, the peak distance (intensity ratio) are in good agreement with comparable measurements found in the literature [8] where the sample preparation was performed in a similar way; in particular, the protocol used in Ref. 7 excludes lysing of red blood cells and removal (washing) of residual dye by centrifuging. The CD4 count of 659 CD4 cells per μl blood is well within the expected range for human blood.

Figure 5(b) shows the measured fluorescent amplitude for each detected cell in sample A as a function of particle speed for a total of 995 detected cells. The detected cells separate into two groups which can be attributed to CD4 lymphocytes and CD4 monocytes. The speed distribution for lymphocytes (diameter ~7 μm) is in good agreement with the theoretical values obtained by solving the Navier-Stokes equation for the given channel geometry. The minimum speed of 75 mm/s reflects the repulsive force a cell experiences in the steep parabolic velocity profile close to a channel wall. Compared to the CD4 lymphocytes (~7 μm), the larger CD4 monocytes (~17 μm) show a narrower speed distribution as flow focusing constrains them to a narrower range of flow speeds in the center of the channel. These results clearly demonstrate the ability of the spatially modulated emission technique to accurately determine the speed distribution of the particles. The ability to monitor the speed of each particle enables simple fluidic handling and true volumetric determination of the analyte.

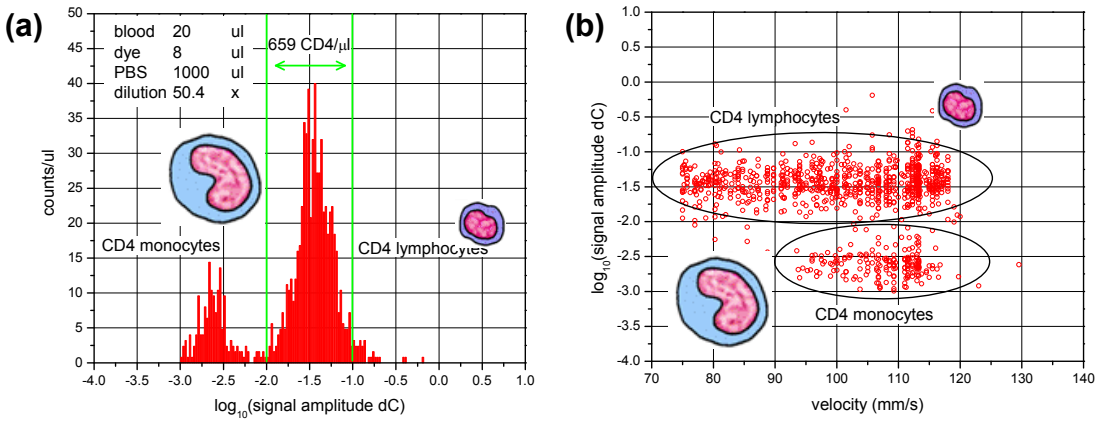

Figure 5: (a) Histogram of detected CD4+ T-lymphocytes for sample A as a function of fluorescent intensity. This constitutes a CD4 count in whole blood (no lyse, no wash, dye: PE) diluted by a factor of 50 obtained by the spatially modulated emission technique. The two peaks identify CD4 lymphocytes (right) and CD4 monocytes (left). Peak distance and relative count rate are in good agreement with published results [8].

(b) Fluorescent intensity as a function of speed for CD4 cells (dye: PE). Compared to the CD4 lymphocytes, the larger CD4 monocytes show a narrower speed distribution as flow focusing constrains them to a narrower range in the flow profile.

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