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Rare cancer cell analyzer for whole blood applications: Microcytometer cell counting and sorting subcircuits

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Abstract

We demonstrate sorting of rare cancer cells from blood using a thin ribbon monolayer of cells within a credit-card sized, microfluidic laboratory-on-a-card ("lab card") structure. This enables higher cell throughput per minute thereby speeding up cell interrogation. In this approach, multiple cells are viewed and sorted, not individually, but as a whole cell row or section of the ribbon at a time. Gated selection of only the cell rows containing a tagged rare cell provides enrichment of the rare cell relative to background blood cells. We also designed the cell injector for laminar flow antibody labeling within 20 s. The approach combines rapid laminar flow cell labeling with monolayer cell sorting thereby enabling rare cell target detection at sensitivity levels 1000 to 10,000 times that of existing flow cytometers. Using this method, total cell labeling and data acquisition time on card may be reduced to a few minutes compared to 30–60 min for standard flow methods.
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Keywords: Rare cells; Cell sorting; Cell counting; Microcytometer; Microfluidic

1. Introduction

The biological changes that are known to be associated with cancer cells encompass the full continuum from mutated or duplicated genomic sequences, shifts in gene expression patterns, as well as altered proteins, all of which have made comprehensive cancer marker analysis difficult. The challenge for practical transfer of the diversity of molecular information being generated in the characterization of cancer to routine clinical practice is the development of reproducible, integrated, and automated methods for measurement. In addition, cancer cells are frequently found in low concentrations and in a background of normal cells. Enrichment of the cancer cells away from background normal cells would dramatically increase sensitivity for early diagnosis, detection of micrometastasis, assessment of minimal residual disease, and monitoring therapy effectiveness. Maximum clinical utility should be requires enrichment sorted cancer cells and rapid analysis of protein, DNA, or mRNA expression alterations from the same specimen.

Efficient integration of a comprehensive cancer cell analyzer should be possible using a microfluidic approach. Individual steps would be performed using fluidic movement and valving logic, with final performance enabled through the design of an integrated microfluidic circuit. This approach offers technology advantages as compared to most macro-scale instrumentation in that it would:

- Minimize user manipulations, training or expertise for operation resulting in more standardized methods that can be adopted at point of care settings.
- Enable complete analysis to be performed on lab cards the size of a credit card, starting with whole blood and incorporating reagents on card.

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- Utilize laminar flow diffusion-based microfluidics to miniaturize the volumes of fluids, such as samples and reagents required to obtain results and thereby reduce cost.
- Provide more rapid results due to microfluidic card design and kinetics that in some cases reduce the time from sample to result from hours to seconds.
- Ensure the containment of waste on card for safe disposal.

Micronics is taking a systematic approach to producing integrated polymeric lab cards for point-of-care cancer diagnostics. For development, the multiple microfluidic functions are optimized separately on lab card subcircuits. These subcircuits include (1) detection and sorting of rare cancer cells from blood; (2) cell disruption, separation, and purification of protein, RNA, or DNA analyte; (3) molecular marker analysis by nucleic acid amplification, proteomic, or genomic arrays; and (4) qualitative or quantitative detection. Once the various subcircuits have been optimized, the final process involves their integration into a fully functioning lab card. Here, we relate the subcircuit methods for counting and sorting cancer cells from whole blood.

2. Cell enrichment approach

Flow cytometry is a well established method for detecting blood cells expressing specific CD antigens. The detection of cancer cell specific antigens in blood has also been investigated [1]. Using flow cytometric properties as a basis, red cell lysis [2], white blood cell enumeration [2], and antibody labeling of cells have been demonstrated within a plastic, disposable, microfluidics-enabled card. However, current flow cytometry methods lack adequate sensitivity to reproducibly detect the disseminated cancer cells that can be as few as 1–10 cells per 10 ml of blood.

Presently, the state of the art in flow cytometry and cell sorting technology uses a hydrodynamically focused core stream which uses two-dimensional focusing of the stream in orthogonal flow to approximate the size of a cell (10 μm). This produces a stream of individual cells in single file, which can be presented to light scatter, fluorescent detector, or image-based cell detection system. This scheme suffers from the significant limitation that the detectors may only assess and direct one cell at a time. To process large quantities of cells for robust point-of-care applications, the fluidic systems must permit movement of numerous simultaneous cells rapidly past the detector. Speeds from one to forty meters per second past the detector are obtained to achieve necessary throughput for some applications. Cells are then partitioned into micro droplets and each micro droplet is charged so that it may be electrostatically deflected into separate bins for sorting.

Higher throughput is necessary to detect rare cells in a reasonable data accession time. The formation of a thin ribbon monolayer of cells within a microfluidic structure is a method of speeding up cell interrogation. In this approach, multiple cells are viewed and sorted, not individually, but as a whole cell row or section of the ribbon at a time. For cell sorting, instead of one cell at time being sorted, a whole cell row containing at least one marked target cell may be diverted upon identification of the target cell signal. The diverted fluid volume may be collected in a storage channel on the plastic card for further processing. The collected, diverted fluid volume can contain both target cells and non-target cells, but the target cells will be present at a much higher concentration than in the original sample through gated selection of only the cell rows containing a tagged rare cell.

In addition, the use of laminar flow dramatically speeds up cell labeling time relative to standard flow methods that rely upon diffusion. This concept has been modeled as shown in Fig. 1. The design of the sample injector is shown in Fig. 2. Cell sorting of fluorescently tagged cells, detected within a monolayer row, are diverted by vacuum fluid diversion using the sorting slit structure as shown in Fig. 3. The design approach therefore combines rapid laminar flow cell labeling with monolayer cell sorting. This approach should permit the collection of rare cell targets at rates up to 1000 to 10,000 times faster than existing sorters.

3. Method

3.1. Fluid movement and microfluidic circuitry

The sample and antibody solutions are moved through the card's channels by the microFlow system, which consists of a controller, pumps (250 and 2300 μl capacity pumps), and a manifold. The microFlow is a commercially available ultra-low-pulse pump system (Micronics) with air, vacuum, forward and reverse pumping capabilities controlled by PC-based software (Fig. 4). Fluidic circuitry is enabled by well-established, proprietary Micronics' technology for valve actuation and design. In a channel on card, fluids can be transported by either air or Fluorinert FC-70 (Hampton Research HR2-797). Fluorinert FC-70 has a viscosity similar to water with approximately 75% greater density, and is not miscible with aqueous solutions, and was used to prevent dilution of the sample and antibody solutions during processing on the plastic card. Lab cards are inserted into a manifold that interfaces with fluid or air lines controlled by the microFlow system.

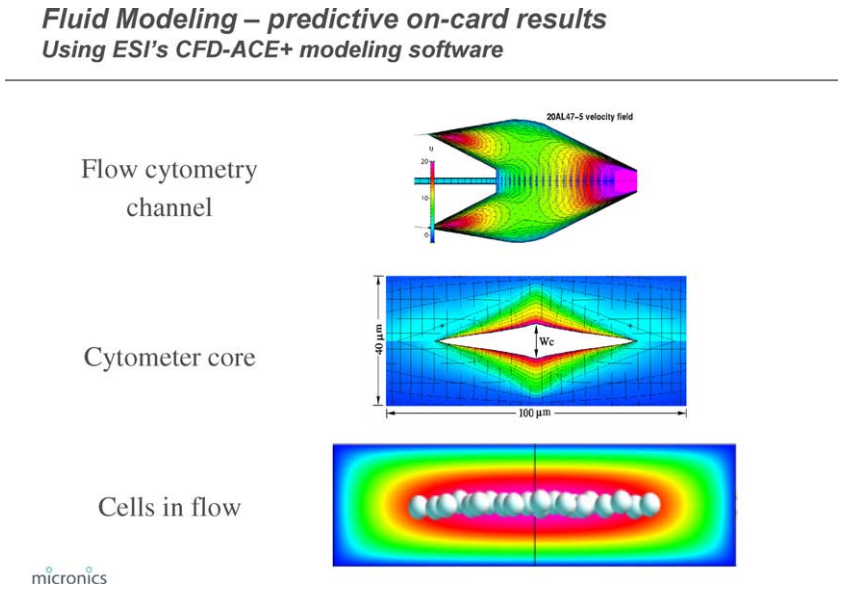

Fig. 1. Fluidic modeling that supports rapid cell labeling and illustrates the concept of monolayer cell interrogation to accomplish rare cell enrichment.

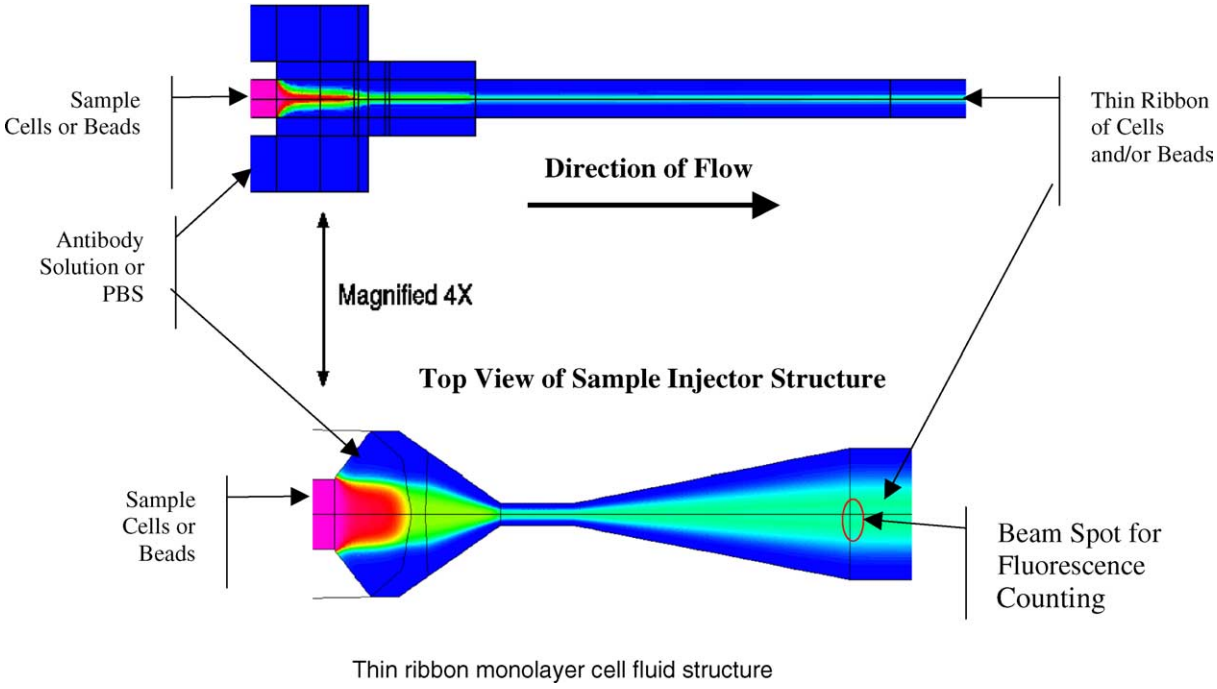

Fig. 2. Thin ribbon monolayer cell fluid structure.

3.2. Lab card design

Fig. 5 shows the microfluidic plastic card used for cell and/or bead counting and sorting experiments. The plastic card consists of a 30 µl sample loop for beads and/or cells, an on-card reservoir holding 400 µl of diluted antibody (if used) or PBS (if no antibody used), an on-card sample injector, labeling loop, view port, and a sorting slit for removal of tagged cells or beads. The card was manufactured using Micronics' laminate prototyping methods. For this, individual layers are laser cut then laminated to form the three-dimensional channels and valves. The card incorporates the antibody labeling, cell monolayer sample injector illustrated previously (Figs. 1–3).

To perform continuous labeling of cell or beads, a small volume of sample was injected into a larger volume containing the antibody using the sample injector. This injector allows the sample to form a thin ribbon within the diluted antibody solution. The ribbon was approximately one cell layer thick. Ribbon width is

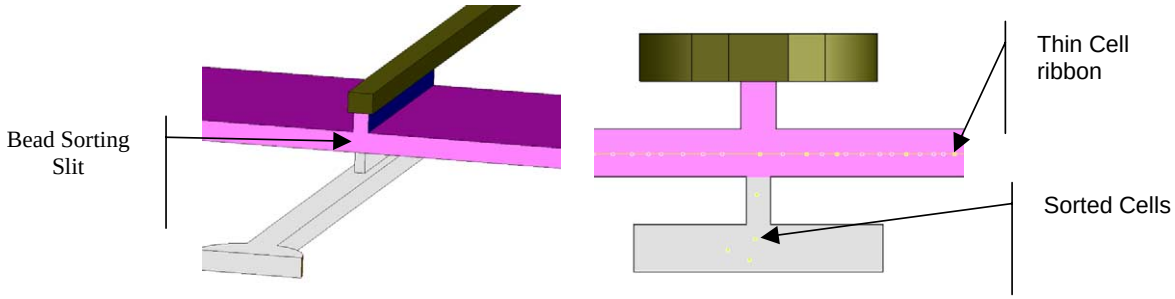

Fig. 3. Cross section views of bead sorting slit structure.

Fig. 4. Micronics' microFlow system.

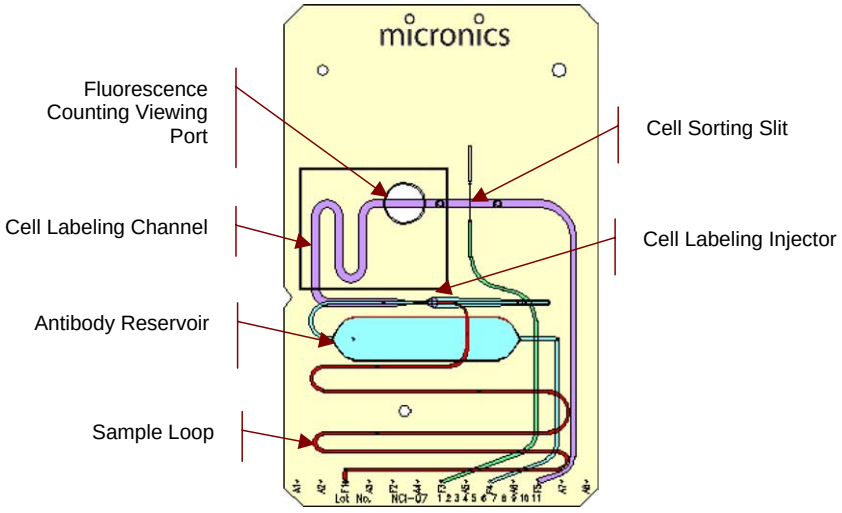

Fig. 5. Plastic microfluidic card for fluorescent labeling of cells and cell sorting.

determined by channel and injector width. For the plastic cards used in these experiments the channels were each 1.5 mm wide. The cell ribbon thickness can be controlled by fluid flow rates and fluid flow ratios. Labeling time was also controlled by fluid flow rates. For the labeling experiments, dwell time in the labeling loop was about 15 s. After optimization, speed was increased and dwell time was maintained using the labeling loop length, which can be increased to provide adequate dwell time.

3.3. On-card optics

The manifold containing the lab card was placed on the stage of a Zeiss inverted microscope (model IM35). The card was illuminated with a BlueSky Research

488 nm laser (model FTEC-488-020-SM00). Charge coupled device (CCD) cameras Andor iXon (model DV 877-BI) and Watec (model LCL-902C, monochrome) were used to view the plastic card through the microscope. The Watec has traditional video output and video from this camera was captured using a National Instruments video capture card (model IMAQ PCI-1409). The data were collected in movie format, which allowed analysis on-the-fly, or could be saved for further analysis at a later time. The analysis portion uses National Instruments LabVIEW (Version 6i) with the Vision add-on (IMAQ Vision for LabVIEW). This software comes with "blob" analysis, which can be configure to recognize bright spots in an area of interest and is used to count beads or cells as they pass through the laser spot.

Counting speed was determined by the camera frame speed and light sensitivity. The sensitivity also was determined by the brightness of the labeled cells or beads. For these experiments, only brightly labeled cells or beads were used. Speed can be increased if the camera reads only a small portion of the field of view. The Watec camera was fixed at 30 frames per second (FPS) while the iXon fps was determined by the configuration number of pixels per line and number of lines as well as readback speed. Maximum FPS for the iXon was 200 FPS. However, faster readback decreases the signal-to-noise ratio, so speed and resolution were a trade-off.

3.4. Fluorescent bead controls

During the initial development phase, we demonstrated the ability to document the fluorescence counting of beads. All beads used for these experiments were obtained from Polysciences. Initial visualization used very bright Fluoresbrite Yellow Green Microspheres. Both 3 μm (calibration grade #17147) and 10 μm (#18140) beads were mixed. These beads are deeply dyed, with nearly the entire bead labeled. Next a medium bright Flow Check FITC 6 μm bead (#24253) was visualized. These beads are not deeply dyed (typically only the outer 10%) and show a ghostlike appearance. Finally, PolyComp beads coated with anti-IgG (#24312) were tagged with the CD4 antibody either on or off card. These beads have both bright and medium bright fluorescence levels. Bead size is unspecified but appears to be about ~6 μm.

3.5. CD4 white blood cell labeling

For feasibility testing, we utilized CD4 to begin with higher cell counts using well established reagents and labeling protocols. The CD4 antibody used was BD Biosciences Pharmingen #557695 AlexaFluor 488 conjugated mouse anti-human CD4. This CD4 antibody is known to stain approximately 15% of the white blood cells in an average blood sample. The CD4 antibodies used were tagged with AlexFluor 488, a dye with similar response to fluorescein conjugates, but more photostable. When illuminated with light of wavelength 488 nm (blue), AlexFluor 488 emits with a wavelength of ~520 nm (green). The Zeiss microscope is outfitted with a filter set that limits transmission to the camera of fluorescent light only, eliminating scatter. No excitation filter was used due to the use of the 488 nm laser. Either a 510 nm 20 db bandpass filter (Chroma Technology Corp #D510/20x—part of filter set 31040) or a 520 nm 40 db bandpass filter (Omega #XF3003) with a beamsplitter (Chroma Technology Corp #505dclp–part of set CZ 716) was used.

The recommended protocol for the CD4 antibodies specifies use of 5 μl antibody reagent to 100 μl whole blood. For on-card labeling tests, the 5 μl of antibody was diluted in 200 μl PBS to provide the sheath volume needed to create the labeling ribbon.

A comparison test was run with white blood cells prepared off card using standard accepted practices. Whole blood was mixed with EDTA and stored at 4 °C. The protocol used 100 μl whole blood and lysed red blood cells with 1.4 ml ammonium chloride. Cells were then washed with PBS and stored at 4 °C until used. When used, cells were resuspended in 100 μl PBS to obtain concentration similar to whole blood.

4. Results

4.1. Fluorescent bead counts on card

Various types of beads, both fluorescent and functionalized, were loaded into the 30 μl sample loop of the plastic card. The antibody reservoir on card was filled with PBS alone (for non-labeled sample), or CD4 mABS diluted with PBS (for on-card labeling of functionalized beads). The sample was pushed with Fluorinert and the antibody reservoir fluid was pushed with PBS if not labeling on card, or Fluorinert for on-card labeling. Fluorinert is used to prevent dilution of the antibody spiked PBS. Various sample and sheath rates were tried. Good labeling appears to occur with a 10:1 antibody spiked sheath:sample flow rate ratio. The sheath flow rate of 1.0 allows for a slow CCD to obtain a good view of each bead or cell. At this flow rate, a sample of 10 μl would take about 2 min to run. The sampling portion of the test takes ~15 s. A faster camera should be able to handle faster speeds.

Fluorescence bead counting was successful with a very accurate correlation to both expected and measured counts, Table 1. Labeled fluorescence PolyComp beads counting (both on and off-card CD4 labeling) was successful but did not show as many counts as expected. The on-card stained PolyComp beads did not show the correct number of counts. This may be due to degrada-

Table 1
Bead and cell counting results

Sample type	Particle size (μm)	Labeled on-card	Sample flow rate (μl/s)	Label flow rate (μl/s)	# Frames/frames per second	Expected number of beads[a]	Observed number of beads[a]	Ratio observed: expected beads
Fluoresbrite (mixed sizes)	3 (10)	No	0.025	1.0	100/33	37 (2.3)	36 (4)	0.97 (1.74)
FITC	6	No	0.1	0.5	100/77	42.8	48	1.12
PolyComp	~6	No	0.1	1.0	100/30	22	20	0.91
PolyComp	~6	1 μl CD4 mAbs: 40 μl PBS	0.1	1.0	30/30	6.6	3	0.45

[a] (50 × 75 μm beam spot).

tion of the laser. The PolyComp beads have a mixture of high and low staining beads. The laser power was measured after this test and showed a fourfold decrease in power. Therefore, it is probable that only the highly stained beads were counted.

Figs. 6 and 7 show examples of the raw video data and the processed data used to determine bead counts.

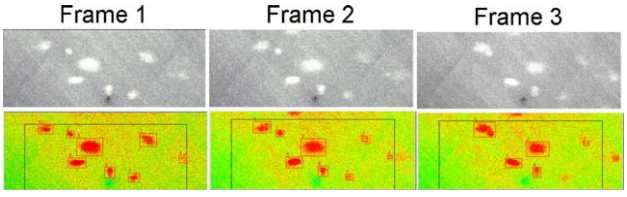

Fig. 6. Frames from video showing the counting of beads and bead movement on card.

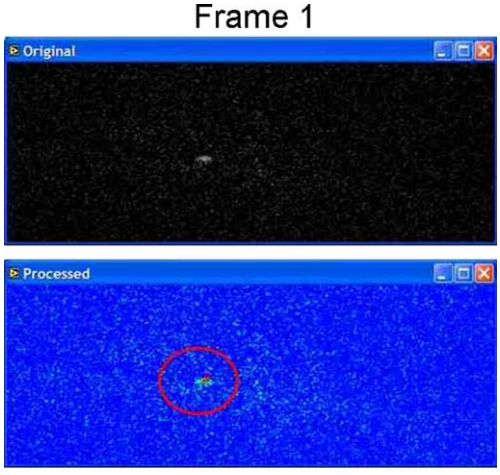

Fig. 7. Frame from video showing the off-card WBCs labeled with CD4. The laser spot is subtracted from the background resulting in a clearer image.

Beads of various sizes could be seen. Based on the optics of current system, beads of ~3 μm were the practical lower limit for this method of counting.

4.2. Fluorescent WBC counts on card

The WBC counts were calculated using average expected values. All blood samples were from the same individual. Table 2 presents a summary of test results. Since only a few cells are expected to be present in the small area of the channel illuminated, the calculated ratios can change with the presence or absence of a single cell. Longer run times and samples from various donors will be tested to provide more statistical significance.

4.3. Antibody labeling on card

Table 3 details the time required for the various steps used to label cells prior to sorting. The normal protocol takes over an hour while the on-card process was completed within 30 s. The volume of reagents is also reduced and the waste can be safely contained on card.

4.4. Sorting/fluorescence gating

The long-term objective is to have automatic fluorescence gating and sorting, however for the feasibility study, the goal was to demonstrate manual sorting of beads. Quantitative results were not pursued at this stage in testing. Beads used either alone or mixed with WBCs were run through the system and captured. Fig. 8 shows the beads moving back into the sorting slit on card.

The sorting volume displaced within the plastic card was defined by the slit width (25 μm), slit length (1500 μm),

Table 2
$CD4^+$ white blood cell counting results

Sample type	Labeled on-card	Sample flow rate (μl/s)	Label flow rate (μl/s)	# Frames/frames per second	Expected number of labeled cells[a]	Observed number of labeled cells[a]	Ratio observed: expected cells
$CD4^+$ labeled white blood cells	No	0.05	0.55	100/91	1.1	2	1.85
White blood cells	1 μl CD4 m Abs:40 μl PBS	0.1	1.0	50/30	3.3	4	1.21

[a] (50 × 75 μm beam spot).

Table 3
Protocol for immunofluorescence staining of human blood cells comparing standard assay to lab card

Assay step	Standard assay conditions	Micronics' formatted assay
Whole blood sample	100 μl	12 μl
Dilution with PBS	400 μl	
Mabs (labeled antibody)	5 μl	0.6 μl (non-optimized)
Dilution with PBS		258 μl
Incubate	20–30 min at 4 °C	20 s at ambient temperature
Centrifuge	5 min	
Remove supernatant	30 s	
Add lysing solution	1.4 ml	500 μl non-optimized
Incubate at room temperature	3–5 min	20 s
Centrifuge		
Remove supernatant	30 s	
Dilutions with PBS	600 μl	
Remove supernatant	30 s	
Dilution with PBS	400 μl	
Cytometric measurement	2–3 min	2–3 min

and slit depth (150 μm). This gave a volume of 5.6 nl to displace entire volume over the sorting slit. A detailed view of the sorting slit is shown in Fig. 3. The current sorting design tends to trap an air bubble. Future plastic card designs will fill the sorting slit structure with liquid without trapping an air bubble. This air bubble did not prevent bead cell capture, as all the beads must pass the open portion of the slit. Current channel design made quantitation of volume of fluid displaced during bead sorting difficult. To overcome this issue, the volume displaced was relatively large. Smaller volumes should be possible when the air bubble is resolved in subsequent card designs.

A 2300 μl capacity pump was used to aspirate fluid at 30 μl/s. The pump was asked to displace about 1–2 μl of fluid. This pump was chosen for the rapid flow rate rather than for small displacement volume.

For cell or bead sorting, the sample flow rate was 0.1 μl/s and the antibody labeling solution flow rate was 1 μl/s. The 5.6 nl displaced sorting volume is expected to contain approximately 4 WBCs. The highest frequency of sorting was measured at 0.91 s per sorting pulse, using the pumps on the microFlow system to sort cells. The actual number of sorted cells was not determinable due to the inability to access the sorted volume. This has been resolved on a subsequent version of the card design.

The thin ribbon cell sorter appears to be a very feasible method of rare cell sorting from whole blood cells. The labeled cells were visualized and their velocity recorded for purposes of determining when to sort a cell. The sorting volume was small and should effectively reduce the number of cells to be analyzed. Cell displacement from a moving stream works well and with some optimization should be able to sample a small volume within the moving stream.

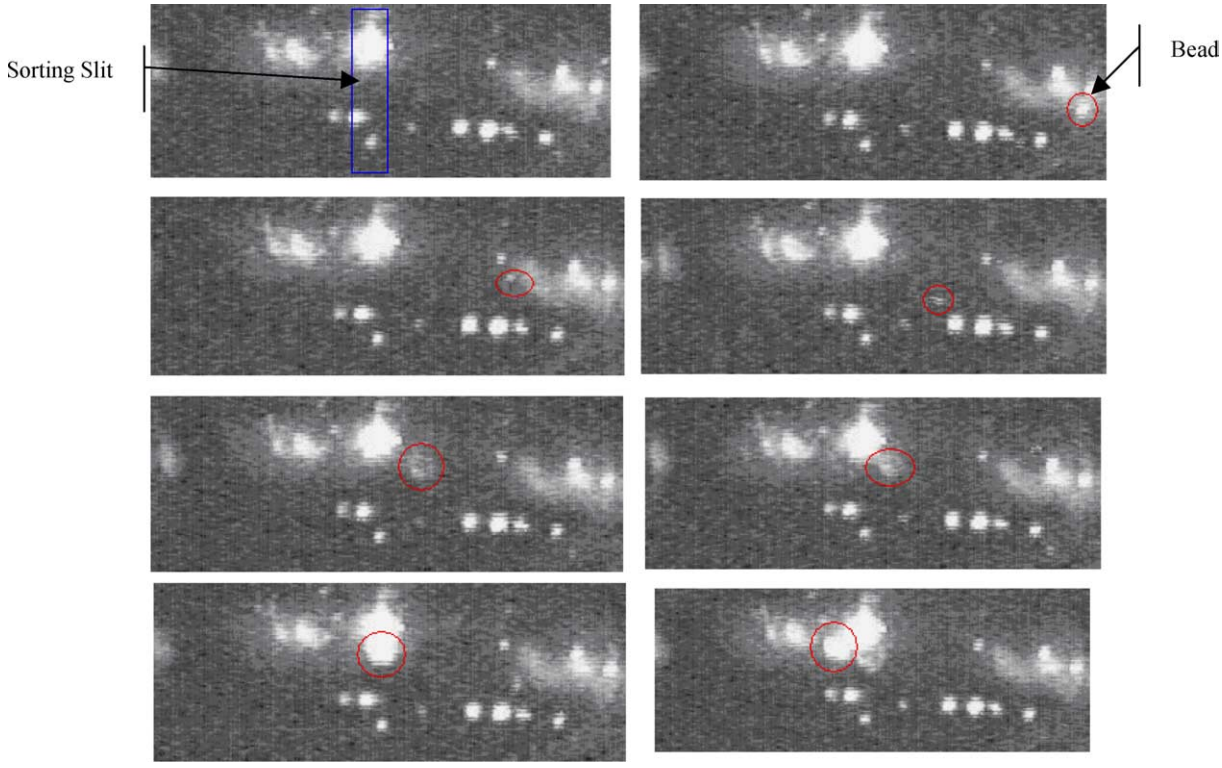

Fig. 8. Bead sorting. Bead moves from right to left of screen. Bead size appears to change as focus changes when particle is sorted below bead thin ribbon plane.

5. Discussion

We have demonstrated the feasibility of an enhanced counting and sorting method using a unique, plastic, microfluidic lab card approach. Based upon our preliminary results, we estimate a 1000-fold increase in sensitivity of detection of rare cells in blood relative to standard flow cytometry. Through increased efficiency and speed of cell labeling and monolayer acquisition, it should be possible to shorten sorting and data acquisition time to just a few minutes compared to an hour for standard flow cytometry. All reactions can be automated, eliminating manual centrifugations and manipulations. This will enable rapid point of care cancer marker analysis.

Although our initial experiments utilized CD4 as an antigen, our ultimate goal is to apply these methods to rare blood cell detection. For example, an important clinical marker used routinely for assessment of hematopoietic stem and progenitor cell numbers critical to bone marrow transplantation is CD34 [3]. CD34 cell enumeration is a marker with established clinical acceptance, but one for which improvements in detection sensitivity is needed. Current reference laboratory methods for $CD34^+$ cell enumeration are time consuming, lack sensitivity, and have poor reproducibility below nine cells per ml [4]. CD34 cells are also the hematopoietic cell that undergoes malignant transformation in chronic myelogenous leukemia (CML). Using the cell sorting methods we describe should enable enrichment of CD34 cells that can then be analyzed at greater sensitivity using integrated lab card subcircuits to confirm other cancer markers specific to CML.

Chronic myeloid leukemia (CML), is a specific cancer, for which antigenic expression, gene translocation, and RNA expression levels have been well characterized and are being utilized clinically. CML is a malignant clonal stem cell disorder considered in a majority of cases to be due to a specific chromosomal translocation between chromosome 9 and chromosome 22 (Philadelphia chromosome) [5,6]. The molecular consequences of this translocation is fusion of the c-ABL oncogene on chromosome 9 to sequences in the breakpoint cluster region (BCR) of chromosome 22 [6,7]. RNA expression of the fused gene results in a chimeric bcr/abl transcript that is processed to produce fusion proteins of varying size, depending on the site of the breakpoint in BCR. The BCR-ABL fusion protein has tyrosine kinase activity that causes malignant transformation [6,7]. Based on this, a drug that inhibits the kinase (Gleevec, imatinib mesylate; Novartis AG, Basel, Switzerland) has been developed, shown to be effective in treating CML patients [7], and received FDA approval. Therefore, for CML patients, detection of the 9; 22 gene translocation, fusion protein, and mRNA expression levels are now being utilized for CML diagnosis and the clinical assessment of therapeutic effectiveness, minimum residual disease, relapse, and disease progression [8,9].

Acknowledgments

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