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A new method for high speed, sensitive detection of minimal residual disease

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Abstract

Investigations of rare cell types in peripheral blood samples, such as tumor, fetal and endothelial cells, represent an emerging field with several potentially valuable medical applications. Peripheral blood is a particularly attractive body fluid for the detection of rare cells as its collection is minimally invasive and can be repeated throughout the course of the disease. Because the number of rare cells in mononuclear cells can be very low (1 in 10 million), a large number of cells must be quickly screened, which places demanding requirements on the screening technology. While enrichment technology has shown promise in managing metastatic disease, enrichment can cause distortions of cell morphology that limit pathological identification, and the enrichment targeting adds additional constraints that can affect sensitivity. Here we describe a new approach for detecting rare leukemia cells that does not require prior enrichment.

We have developed an immunocytochemical assay for identification of leukemia cells spiked in peripheral blood samples, and a high-speed scanning instrument with high numerical aperture and wide field of view to efficiently locate these cells in large sample sizes. A multiplex immunoassay with four biomarkers was used to uniquely identify the rare cells from leukocytes and labeling artifacts.

The cytometer preserves the cell morphology and accurately locates labeled rare cells for subsequent high resolution imaging. The sensitivity and specificity of the approach show promise for detection of a low number of leukemia cells in blood (1 in 10 million nucleated cells).

The method enables rapid location of rare circulating cells (25M cells/min), no specific enrichment step, and excellent imaging of cellular morphology with multiple immunofluorescent markers. The cell imaging is comparable to other imaging approaches such as laser scan cytometry and image flow cytometry, but the cell analysis rate is many orders of magnitude faster making this approach practical for detection of rare cells.

Keywords

Rare cell; minimal residual disease; acute lymphoblastic leukemia; image cytometry

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Introduction

Rare cell cytometry aims at detecting, enumerating and characterizing rarely occurring cells. Although no universally accepted definition of “rare cell” exists, the general consensus is that cells occurring below the frequency of one cell in the background of a million leukocytes require special processing for reliable detection. Assays based on detection of rare cell types in peripheral blood (PB) samples represent an emerging field with several potentially valuable medical applications. PB is a particularly attractive body fluid for diagnostics, as hematologic spreading is a common mechanism in the dissemination of disease, and its collection is a minimally invasive procedure that can be repeated throughout the course of the disease.

Most efforts in rare cell detection target detection of circulating tumor cells in PB of patients with solid tumors, and a several approaches have been used to detect these cells (1–6). Most rely on cell enrichment through targeting the epithelial cell adhesion molecule (EpCAM) on the tumor cell membrane which can lead to loss of sensitivity (7) and is not applicable to EpCAM-negative tumors.

For patients with childhood acute lymphoblastic leukemia (ALL), precise measurements of leukemia cells in bone marrow or blood specimens enable the quantification of minimal residual disease (MRD) and provide a strong predictor of relapse (8–11). Many protocols now use MRD levels to adjust intensity of treatment. MRD is typically measured in bone marrow aspirates, a procedure that usually requires general anesthesia in children. The most commonly used methods to measure MRD in patients with ALL are flow cytometry and polymerase chain reaction (12). Both approaches rely on abnormal cell profiles in ALL cells and each has advantages and disadvantages (13–15).

If MRD could be measured in peripheral blood instead of bone marrow, monitoring of MRD could be done at closer intervals throughout the course of treatment. Several groups have studied MRD in blood using both sensitive flow cytometry and PCR techniques (16–20). These studies all agree that the percentage of leukemia cells to mononuclear cells in blood parallels the percentage in bone marrow. However, while these percentages are comparable in T-lineage ALL, they are substantially reduced in peripheral blood for B-lineage ALL.

In this paper, we describe a new method for measurement of MRD with improved sensitivity and specificity that involves no specific enrichment step. Leukemia cells are located on a planar surface by a high speed cell scanner that was developed to locate circulating tumor cells (21), (22). Multiple biomarkers on located objects are subsequently imaged in an automated digital microscope (ADM). The high resolution ADM imaging results in an improvement in sensitivity over commonly used methods. Without limiting the ADM imaging to only objects located by the scanner, the imaging time would be prohibitively long (23). The scanner collection optics are built with fiber array scanning technology (FAST) that enables a wide field-of-view (76 mm) at a high numerical aperture (NA=0.66) (21) at the expense of resolution. The wide field of view eliminates the need to rapidly move high inertia collection optics, but the low resolution requires subsequent imaging with automated digital microscopy ADM.

Materials and Methods

Sample Preparation

Blood sample processing follows our previously described multiplex-labeling protocol for detection of circulating tumor cells from solid tumors (21) with some changes such as antibodies relevant to detecting ALL cells. Blood from healthy human volunteers was drawn

into tubes containing anti-coagulation reagent ethylenediaminetetraacetic acid (EDTA) (BD Biosciences). The erythrocytes were lysed with 5X volume of isotonic ammonium chloride buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4) at room temperature for 5 min. After centrifugation (300 RCF for 5 min at room temperature), the remaining white cell pellet was washed and resuspended in PBS. Mononuclear cells were counted in a small volume by using trypan blue exclusion. Test samples were prepared by spiking 50–200 Nalm6 cells in 3-million leukocytes. The cells were spread on a section of large (10.8 cm × 7.6 cm) custom-designed adhesive slides (Marienfeld, Bad Mergentheim, Germany) using pipettes and incubated at 37°C for 40 min in humidified incubator to allow cell attachment. The cells were fixed by adding a solution of 2% paraformaldehyde in PBS. After decanting, ice-cold acetone was added, and the slide was moved to –20°C freezer for 5 min. The slide was then rinsed in PBS 1X and blocked with 20% human AB serum (Sigma H-4522) in PBS at 37°C for 20 min. After blocking, the solution was decanted and the slide underwent a series of antibody staining and incubations at 37°C, including primary (1hr), secondary (1hr) and tertiary antibody (30 min). Rinses in PBS were performed twice for 3 min each between antibodies. The antibody mixtures were prepared in 20% human AB serum. A list of the four antibodies (CD10, CD66b, CD19 and CD58) used in the assay with their vendors and working concentrations are provided in Table 1. To reduce dye aggregates, all staining solutions were filtered through 0.22 μm syringe filters followed by high-speed centrifugation and removal of the sediment pellets. After labeling, substrates are mounted in aqueous medium FluorSave™ reagent (Calbiochem) and left in the dark overnight for the mounting medium to cure before the edges are sealed with nail polish.

The protocol was modified for specific experiments. In experiments to evaluate the detection sensitivity of the FAST cytometer and characterization of false positives, CellTracker Blue was used to pre-stain cells to enable detection without immunolabels using microscopy. In these experiments, the labeled antibodies for CD10 and CD66b were used for cytometer detection and the nuclear stain DRAQ5 (Biostatus Ltd) was added for cell identification, as it has no spectral overlap with CellTracker Blue. In the specificity experiments using all four antibodies, no nuclear stain was used with the CellTracker Blue. Control experiments using non-spiked sample of 3 million leukocytes from the same donor included four-antibody staining and nuclear counterstaining with 4',6-diamidino-2-phenylindole (DAPI) (D-21490, Molecular Probes). For the clinical assay, DAPI will be used to stain the nucleus as CellTracker Blue is absent.

Cultured cells (Pre-B cell line Nalm-6) were used to model ALL cells. Nalm-6 cells (ACC 128, purchased from DSMZ, Germany) were cultured with RPMI1640 supplemented with 10% FBS at 37°C in a 5%–CO₂ incubator. The Nalm-6 cells are pre-stained with CellTracker Blue to provide a label-independent method of identifying the cells that is brighter than the immunofluorescent labels. CellTracker is added to a flask with one to two million Nalm-6 cells 30 min prior to harvest, as per manufacturer's protocol. For evaluating the assay, 50–200 Nalm-6 cells labeled with CellTracker are spiked into 3 million leukocytes from blood of a healthy donor. All donors gave their written, voluntary, informed consent to participate, and the acquisition was approved by the institutional review board at the Palo Alto Research Center.

FAST Cytometer

In the approach described here, ALL cells are located with a separate scanning instrument, the FAST cytometer, that targets CD10⁺CD66b[–] objects without subcellular resolution. Objects are subsequently imaged in an automated digital microscope (ADM) at higher resolution for measurement of expression levels of biomarkers used to identify ALL cells. The scanning instrument employs laser printing optics to scan at high speeds with a 488 nm laser focused to a 10 μm spot. The laser reflects off one of the 10 facets of a spinning

polygon mirror causing it to sweep 76 mm across the substrate at a speed of 10 m/sec. At this rate a 64 cm² substrate containing 25 million nucleated blood cells can be scanned in 60 seconds. The substrate is supported on a stage that is programmed to raster the scans with an 8 µm separation. The start of each scan is sensed by a custom start-of-scan circuit. The circuit uses a split detector to sense the movement of the laser beam across a start point during each scan and delivers an electronic pulse that triggers data acquisition.

The key enabler in the instrument is collection of emission by an asymmetric fiber optic bundle that is wide (76 mm) and thin (1 mm) on one end and round (10 mm diameter) on the other. The bundle contains over 40,000 fifty-micron diameter fibers and is placed in close proximity (1 mm) to the fluorescence to maximize capture of the emission. The numerical aperture of the glass bundle is 0.66. Fluorescence transmitted from the 1 cm² round end is collimated within 10° and passed through a dichroic mirror that splits the beam into two wavelength regions. Fluorescence is filtered by double cavity emission filters centered at the two wavelength regions (525 nm and 580 nm) and detected by photomultiplier tubes. These filters have a contrast of 10⁸ for incident light within 10°, which is adequate to block scattered laser light. The photomultiplier output is amplified and passed through low-pass filters before digitization in a high-speed A/D capture card (21).

Scan times are used to determine the location of the laser beam to an accuracy of 40 µm, and the locations are referenced to alignment marks placed on the corners of the substrate. These marks are located by the ADM and used to transform the object's coordinates to the ADM coordinate system. The location accuracy is well within the field of view of a 20× objective lens so each object can be automatically imaged. This "optical enrichment" substantially reduces the number of exposures at high resolution. A wavelength comparison method is used to eliminate autofluorescing particles, the primary source of false positives in the FAST scan as well as granulocytes. The method involves measuring emissions at two different wavelengths, one at the target emission wavelength (580 nm) and the other at 525 nm, a wavelength intermediate between the target emission and the laser excitation (488 nm). Because we have observed leukocyte autofluorescence to be more intense at wavelengths closer to the excitation (24), the ratio of the intermediate wavelength intensity to the target wavelength intensity is greater than for labeled objects. Using the ratio, autofluorescing particles are eliminated using software filtering. With this filtering, 99.8% of the autofluorescent objects are eliminated as well as most of the granulocytes. Without this filtering, the number of autofluorescing objects for microscope imaging would be prohibitively large.

Granulocyte filtering requires an additional label. For ALL cells, the FAST detection target is common acute lymphocytic leukemia antigen (CALLA or CD10), which is highly expressed in more than 90% of ALL cases (25). CD10 is labeled with a tertiary antibody (NeutrAvidin-R-PE) which emits at 580 nm, to increase its signal. Because CD10 is weakly expressed in granulocytes (26–27), CD66b, is used as a negative marker to distinguish them. The CD66b is labeled with Alexa 488, which emits at 525 nm. During data analysis from a FAST scan, software filtering uses the CD66b:CD10 ratio to remove granulocytes along with the autofluorescent objects.

Measuring marker expression levels

Images of cells are analyzed with an internally developed software tool that measures expression levels of different proteins within a cell. Micrographs of fluorescence from antibody labels are stored in a database. Images are displayed as a red, green and blue composite typically with red showing fluorescence from the CD10 label, blue showing fluorescence from the nuclear label and green showing fluorescence from labels of additional biomarkers. The intensity and contrast of each color can be independently

adjusted. A zoom function enables image magnification. The tool can display compressed images (JPEG) or uncompressed loss-less images (PNG), and analysis is typically done with uncompressed images that display sharper features.

A circle image is superimposed on the micrograph and can be moved across the micrograph with a mouse. The diameter of the circle is adjustable with the mouse buttons. The peak and average fluorescence within the circle for each of the three displayed colors is calculated from the individual pixel values and circle area. The tool enables measurement of multiple areas of the cell and provides an average expression value.

Microscopy for sensitivity comparison

The detection sensitivity was tested through comparison with automated digital microscopy (ADM). Healthy donor blood underwent erythrocyte lysis and 3 million leukocytes per test sample were spiked with Nalm-6 cells and plated on a microscope slide on an area of 800 mm². Samples were scanned by the FAST cytometer, and target objects (CD10⁺ CD66b⁻) were subsequently imaged by ADM at 20× magnification for positive identification.

Independently, Nalm-6 cells labeled with CellTracker Blue were located by an ADM. Here the samples were scanned at a much slower rate and lower numerical aperture (0.2) by ADM at 4× magnification by stepping the field-of-view (3.7 mm²) across the sample. Cells stained with CellTracker were located by image analysis filtering, and these objects were subsequently imaged with a 20× objective for positive confirmation. The locations of the cells detected with the ADM were compared to locations of cells detected with the FAST cytometer.

Results

For the ALL MRD assay, we used CD10 as the detection target for the FAST cytometer, as CD10 is highly expressed in more than 90% of ALL (25). In normal blood, low levels of CD10 are also expressed in granulocytes (26–27), and most granulocytes are eliminated by CD66b staining. Non-cellular false positives, such as dye aggregates, are fluorescent objects with no nucleus or cell structure and are eliminated during high magnification review. Other leukocytes are differentiated by CD19 and CD58.

To evaluate the assay, Nalm-6 (CD10⁺, CD66b⁻), a pre-B ALL cell line, was used to model B-lineage ALL cells. Blood samples containing 3 million leukocytes were spiked with 50–200 Nalm-6 cells pre-stained with CellTracker Blue. CellTracker provides positive identification of Nalm-6 cells upon review of ADM images. To evaluate FAST assay sensitivity, the locations of CD10⁺CD66b⁻ Nalm-6 cells detected by the FAST cytometer were compared to locations of Nalm-6 cells found by ADM using the CellTracker label. In 4 separate spiked samples, FAST scanner located on average 84% (±11%) of Nalm-6 cells detected with the ADM (Fig1A). Typically 95% of leukocytes remain on the substrate after sample preparation. The 3-step labeling results in emission from 95% of Nalm-6 cells to be 10-fold above the detection limit of the FAST cytometer. Some of this margin is lost without the amplification of 3-fold labeling.

With the CD10 and CD66b labeling, the FAST cytometer separated Nalm-6 cells from B lymphocytes and most granulocytes, two major sources of false positives, as shown in Fig. 1B. The frequently encountered problem of bright autofluorescence from human eosinophils is not an issue with this assay, as this autofluorescence is lower than the acquired fluorescence due to charge-related, non-specific binding of the dye. Consequently, acquired Alexa 488 on eosinophils is brighter than the CD10 label, and they are eliminated by the scan filtering. In four spiked samples, the FAST cytometer detected on average 22 (±11) false positives. On

average 7.6 (± 6.3) of these were non-cellular false positives that are readily distinguishable from intact cells by morphology and the absence of nuclear labeling (Fig. 2). The remaining (on average 14.4) false positives had leukocyte morphology, and additional markers are required to uniquely identify the Nalm-6 cells from these false positives using expression level analysis from high resolution imaging (Fig. 3).

Several markers can be used to differentiate ALL cells from CD10⁺ cells (28). While the number of these markers is large, the ALL phenotype can be identified at diagnosis when the concentration is higher using flow cytometry, and only relevant markers used for the FAST cytometer assay. For this Nalm-6 assay, two additional markers, CD19 and CD58, were included to confirm B-cell origin and distinguish Nalm-6 cells from the other CD10⁺CD66b⁻ cells (Fig. 4). Expression levels of these markers were measured on high resolution microscope images of cells located with the FAST cytometer (Fig 3).

High levels of CD19 expression were measured on Nalm-6 cells unequivocally confirming their B-lineage, while nearly all of CD10⁺CD66b⁻ false positives were differentiated from Nalm-6 cells by absence of CD19 staining (Fig. 4B). In four spiking experiments an average of 5.9 granulocytes (± 2.2) among 3 million leukocytes were indistinguishable from Nalm-6 cells by CD10 and CD66b but were differentiated by CD19. On average, less than one of these false positive cells was indistinguishable from Nalm-6 cells and probably represent very rare B-cell progenitors in normal peripheral blood (29). For further differentiation of Nalm-6 cells, we used CD58 (LFA3), a useful marker for identifying B-lineage ALL as it expresses in B-lineage ALL cells at higher levels than in normal CD19⁺/CD10⁺ B cell progenitors in about 50% of patients (28,30–32). In flow cytometry analysis, the Nalm-6 cells displayed a phenotype of CD10⁺CD66b⁻CD19⁺CD58^{hi} (Data not shown). As shown in Fig. 4B, the expression level of CD58 in these false positives, which are presumed to be normal B cell progenitors, is significantly lower than expression in spiked Nalm-6 cells.

Discussion

In this paper, we describe testing the FAST cytometer to detect B-lineage MRD in blood. The FAST cytometer was shown to detect 84% of the model B-ALL cells (Nalm-6) found by automated digital microscopy. Of the Nalm-6 cells that were detected by microscopy but missed by the FAST cytometer, ~45% were eliminated by the filtering due to close proximity of granulocytes containing the negative marker, CD66b, ~45% were in the vicinity of high autofluorescence, and 10% were apoptotic with lower expression levels of CD10. We plan to explore reducing the density of leukocytes on the substrate to increase the spacing from granulocytes and investigate the algorithm for removing autofluorescent objects to reduce the elimination of B-ALL cells by software filtering. While a 3-step labeling process provides adequate margin between the dimmest CD10 emission on the Nalm-6 cells and the FAST detection threshold, brighter labeling for the CD10 would minimize cells filtered away due to proximity to leukocytes stained with CD66b or autofluorescence.

The FAST cytometer enables “optical enrichment” by locating rare cells. Because the instrument lacks subcellular resolution, detected objects are subsequently imaged in an ADM at much higher resolution for quantitative analysis of antigen localization and cell morphology. This “optical enrichment” substantially reduces the number of exposures at high resolution and makes subsequent imaging by an ADM practical. The FAST cytometer locates fewer than one object that needs further analysis in 10⁵ leukocytes. This level of specificity leads to fewer than 500 objects in 10 ml of blood that require microscopic imaging. At five seconds per image, this requires about 40 minutes of imaging. This time can be reduced by faster autofocus. Note this high level of specificity is enabled by rapid

elimination of granulocytes expressing CD66b during the software filtering of the low resolution FAST cytometer images. The large number of granulocytes makes high speed imaging and filtering essential and could not be done practically with the subsequent high resolution imaging using the ADM. While we were able to design a gate to achieve this specificity in the samples tested containing 3 million leukocytes, this gate needs to be tested on larger sample sizes, where the intensity distribution of the labeling will be wider, and with actual B-lineage ALL cells, which may have a wider distribution of biomarker expression levels than the Nalm-6 cell line. Patient samples are also needed to test whether natural heterogeneity of blood or diseases could cause interference with detection of leukemic cells; however, markers that can distinguish leukemic cells from their normal counterparts do exist and have been proven to be clinically useful with flow cytometry (13–14,33).

High resolution imaging of the cells is key to improving specificity beyond 10^{-5} . Two other approaches, imaging flow cytometry and laser scan cytometry, also enable high resolution image acquisition. Imaging flow cytometry combines the visual power of microscopy with the speed and sensitivity of flow cytometry (34–35) through employing a precise method of electronically tracking moving cells so that up to 12 images of each cell can be acquired. The imaging system is high resolution, comparable to $20\times$ – $60\times$ microscopy. The imaging allows evaluation of cell morphology and marker localization while scanning up to 1000 cells per second. With laser scan cytometry (LSC) a focused laser beam is raster scanned across a sample, and emission imaged at high resolution. The use of collection optics with a high numerical aperture and a photomultiplier enables very sensitive detection comparable to flow cytometry. LSC instruments are available with multiple excitation lasers and photomultipliers, and four labels can be measured simultaneously. The high resolution imaging enables quantitative analysis of antigen localization and cell morphology (36–37). The throughput of LSC, however, is limited by the scan speed ($0.4\text{ mm}^2/\text{sec}$ per color channel) and field of view. While both of these approaches are used in several cell analysis applications, their throughput is insufficient to enable use for rare cell detection.

The leukemia assay uses a combination of dyes and Qdots to label three markers, CD10 (PE), CD19 (Alexa 647) and CD58 (Qdot800), to phenotype the B-lineage ALL. The additional marker, CD66b (Alexa 488), is used to identify granulocytes. The labels are chosen to minimize overlap and bleed-through of label emissions without compensation. The assay was adapted from a 5-marker assay used to identify circulating tumor cells (CTCs) and measure biomarker expression (38). The two additional labels included in the 5-marker assay are Alexa 750 and Qdot 705, which could be used to label additional biomarkers in a leukemia assay. In a clinical assay that does not contain CellTracker Blue, a nuclear stain, DAPI, will be included to help identify non-cellular fluorescent objects and non-specific immunostaining. Additional markers could be included to further increase the multiplex level, but compensation of bleed-through will likely be needed. Here Qdots with narrower emission bands could be advantaged; however, replacing neutravidin PE for CD10 labeling will need to be tested. While the brightness of Qdot 655 is equivalent to PE, Qdot655 is larger than PE, which may lead to lower amplification in the 3-step labeling.

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Figure 1.

A) The sensitivity for detecting Nalm-6 cells by FAST as compared to standard ADM approach. **B)** B-ALL (Nalm-6) cells are located by CD10⁺CD66b⁻ staining, and the vast majority of granulocytes (CD10^{lo}CD66b⁺) and B-lymphocytes (CD10⁻CD66b⁻) are sufficiently separated that they can be eliminated by FAST scan filtering.

Figure 2.

Three types of false positives and Nalm-6 cells found by CD10(red)/CD66b(green) FAST scanning (highlighted center in the images). Dye aggregates can be distinguished from cells (both FPs and TPs) during review by their morphology (upper panel) and lack of nuclear DNA staining (blue, bottom panel).

Figure 3.

Images of 3 biomarkers: CD66b, CD19 and CD58, show expression level differences between Nalm-6 cells (column 1) that model ALL cells and false positives from normal B-lineage cells (column 2), non B-lineage cells (column 3) and granulocytes (column 4). All of these cells are CD10+ and are detected by the FAST cytometer. The expression level for the biomarker in each image is measured by image analysis and appears beneath the image.

Figure 4.

Separation of Nalm-6 cells from mononuclear blood cells using 4 markers. **A)** Several false positive cell types are included in the CD10^{hi}CD66b⁻ gate (solid line rectangle). **B)** Cell types are further distinguished by CD19 and CD58 markers. B-ALL (Nalm-6) cells here show a CD10^{hi}CD66b⁻CD19⁺CD58^{hi} phenotype that can be defined by a gate.

Table 1

Sources and concentrations of antibodies and labels used in the assay in this study.

Primary (Clone or Cat. #, Vendor)	Working conc.	Secondary	Working conc.	Tertiary	Working conc.
CD10 (HI10a, BD)	10 ug/ml	Goat anti-Mouse IgG1-Biotin (IVTG)	6.6 ug/ml	NeutrAvidin-PE, (IVTG)	10 ug/ml
CD66b (G10F5,BD)	0.125 ug/ml	Goat-anti-Mouse-IgM-Alexa488 (IVTG)	5 ug/ml		
CD19 (HD237, Santa Cruz Biotech)	4 ug/ml	Goat-anti-Mouse-IgG2b-Alexa 647 (IVTG)	10 ug/ml		
CD58 (IC3, BD)	5 ug/ml	Rat-anti-Mouse-IgG2a (Southern Biotech)	5 ug/ml	Goat-anti-Rat-Q800 (IVTG, custom)	20 ug/ml
CellTracker Blue (C2110, IVTG)	10 uM				

(IVTG=Invitrogen).