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Isolation and analysis of rare cells in the blood of cancer patients using a negative depletion methodology

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Abstract

A variety of enrichment/isolation technologies exist for the characterization of rare cells in the blood of cancer patients. In this article, a negative depletion process is presented and discussed which consists of red blood cell (RBC) lysis and the subsequent removal of CD45 expressing cells through immunomagnetic depletion. Using this optimized assembly on 120 whole blood specimens, from 71 metastatic breast cancer patients, after RBC lysis, the average nucleated cell log depletion was 2.56 with a 77% recovery of the nucleated cells. The necessity of exploring different anti-CD45 antibody clones to label CD45 expressing cells in this enrichment scheme is also presented and discussed. An optimized, four-color immunofluorescence staining is conducted on the cells retained after the CD45-based immunomagnetic depletion process. Different types of rare non-hematopoietic cells are found in these enriched peripheral blood samples and a wide range of external and internal markers have been characterized, which demonstrates the range and heterogeneity of the rare cells.

Keywords

Circulating tumor cells; Flow cytometry; Immunocytochemistry; Immunofluorescence; Immunomagnetic separation; Tumor-related cell marker

1. Introduction

The concept of isolating and characterizing rare cells in clinical blood specimens is not new. However, as our knowledge of various biological functions and markers continues to increase, demands continue to increase on the performance of isolation and characterization technologies for known and presumed rare cells. In addition, the promise of personalized medicine supplies additional pressure to develop technology that is robust, accessible, and relatively low cost. With respect to performance standards, beyond typical performance

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standards as recovery, purity, and reproducibility, there is growing demands to extend these performance standards to lower frequency of rare cells.

An example of the evolution of rare cell isolation in clinical samples is the search for circulating tumor cells, CTCs. The classical definition of the CTC, which evolved in the late 1990s, is a cell which is negative for a highly-conserved hematopoietic cell marker, CD45, positive for internal structural proteins that are consistent with epithelial cells, cytokeratins (CK), and positive for an epithelial cell surface marker, epithelial cell adhesion molecule (EpCAM) [1]. Using this definition, a number of technologies designed for the isolation of pure CTC populations have been developed in recent years. The most widely known isolation technology is the CellSearch system, which has been extensively utilized in clinical research and received FDA approval as a tool in CTC studies [2,3]. CellSearch uses immunomagnetic anti-EpCAM antibodies which allows for the capture of EpCAM positive cells for further staining and CTC enumeration. In the wake of CellSearch, other technologies have attempted to refine the capture-based approach, including microfluidic designs to increase the interactions between cells and capture mechanism [4,5]. These approaches are based on the definition put forth above, namely that the CTC would have EpCAM on the cell surface, and can be generalized as positive selection techniques.

However, the very advantage of separating cells by one surface marker is also a shortcoming. The EpCAM capture-based approach can only positively select those cells that have, in this case, sufficient surface expression of EpCAM to facilitate the separation/isolation. However, the variation in phenotypes of CTCs, not just across different cancers, but even within the same patient, has begun to be noted by many sources [1], and positive selection with a single marker risks not identifying all of the CTCs of interest. In order to study a variety of phenotypes in not only CTCs, but other rare cells associated with cancer, we have developed a negative depletion approach to remove what are assumed to be normal hematopoietic cells, thereby providing a highly enriched cell suspension upon which further cellular characterization can be conducted.

In this report, we present our most current technique for the enrichment and characterization of rare cells from the blood of cancer patients. Our cell enrichment technique is based on the removal of red blood cells by chemical lysis and the magnetic depletion of normal hematopoietic cells labeled with an anti-CD45 antibody/magnetic nanoparticle complex. This procedure has been refined over a period of years and is presented here in its current form [6,7]. Along with the detailed protocol for the isolation of non-hematopoietic cells we consider how CD45 labeling can affect the subsequent analysis of the isolated populations, and demonstrate using flow cytometry the importance of proper antibody clone selection between the isolation and analysis steps. Our cell characterization technique is primarily based on an optimized, four-color immunofluorescence staining protocol applied to the rare non-hematopoietic cells, which originate from patient blood specimens and are enriched with our CD45-based magnetic depletion. On these cells, a wide range of external and internal markers have been characterized, either previously published, or in this report, which demonstrates the range and heterogeneity of the rare cells. These markers include extracellular markers, such as EpCAM, epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2 (HER2), N-cadherin (N-CAD), smoothened (Smo), etc., as well as internal targets, such as CK, Gli1 and a DNA damage indicator—gamma-H2AX.

2. Materials and methods

2.1. Sample collection

Summarized in this report, one hundred twenty (120) samples were collected from seventy-one (71) breast cancer patients enrolled in an institutional review board (IRB) approved

protocol. All patients gave their informed consent to participate in the study. Patients enrolled in this study must be histologically confirmed breast cancer stages I–IV with either estrogen receptor (ER) positive or progesterone receptor (PR) positive tumors that are not HER2 overexpressing or $ER^{-}PR^{-}$HER2 non-overexpressing (triple negative). In stages I–III, patients are eligible if they have not received any treatment (including hormonal therapy) for breast cancer other than surgery prior to baseline measurement. In stage IV, eligible patients may be previously treated for metastatic disease ≤2 systemic treatment (excluding hormonal therapy) and about to begin a new course of hormonal treatment or chemotherapy. For patients in stages I–III, blood is collected prior to treatment on the first day of adjuvant/neo-adjuvant treatment, on day 1 of the second cycle of treatment, and then 4 weeks after the final dose of adjuvant treatment. For patients in stage IV, blood is collected prior to treatment on the first day of a new treatment, on day 1 of the second cycle of treatment, and then four weeks after the administration of the final chemotherapy/hormone therapy given up to the time of documented disease progression.

In addition, peripheral blood was obtained from patients with a diagnosis of squamous cell carcinoma of the head and neck undergoing surgical resection for their disease and whom had not been previously treated for this disease. The procedure was IRB approved and informed, written consent was obtained from all subjects. Blood samples were collected from a venous line, either immediately prior to, and/or post, surgery and processed within twenty-four hours after procurement.

Normal blood was obtained through an IRB-approved purchase of source leukocytes from the American Red Cross (Columbus, OH). The breast cancer cell line MCF7 and head and neck cancer cell line SCC4 were procured from ATCC (Manassas, VA) and grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and non-essential amino acids. Cells were maintained at 37 °C in a humidified, 5% CO_2 atmosphere and harvested by washing with phosphate buffered saline (PBS) and subsequently incubating with Accutase (Innovative Cell Technologies, San Diego, CA) at 37 °C until the cells detached from the culture flask. Cells were washed with PBS prior to enumeration for each experiment.

2.2. Negative immunomagnetic cell separation

The negative immunomagnetic cell separation method consists of three parts: (1) red blood cell (RBC) lysis, (2) immunomagnetic labeling targeting CD45, and (3) magnetic separation. The flow chart of the overall process is presented in Fig. 1. Typically, the concentration of RBCs is approximately 1000 times the concentration of peripheral blood leukocytes (PBLs), and the PBL concentration is more than 1 million times the concentration of rare non-hematopoietic cells. To separate out rare non-hematopoietic cells with high purity and low cell loss, the protocol described below is an attempt to minimize the number of cell processing steps while depleting the maximum number of white blood cells from the sample and recovering the maximum number of CD45 negative cells.

2.2.1. Red blood cell lysis—To remove RBCs, a chemical lysis step is employed on the blood. Ammonium chloride lysis buffer is used, which exploits the RBC's relative weakness to osmotic pressure compared to other cell types. Previous spiking studies have demonstrated the superior recovery of spiked MCF7 cancer cells by chemical lysis in comparison to the Ficoll–Hypaque density gradient separation technique [8].

Approximately 7–17 ml of peripheral blood were collected in Vacutainer tubes (#366643, BD Biosciences) for CTC enumeration and processed within 4 h of blood draw. The lithium heparin coating is beneficial in preventing clumping of the white blood cells prior to analysis. The RBC lysis procedure was carried out as previously published [8]. Briefly, the

blood is mixed with 1× lysis buffer at a ratio of 1:20 to the total volume. After 5 min incubation, the cell lysate is centrifuged, supernatant discarded, and the cell pellet is collected. The cells are subsequently washed with labeling buffer prior to immunomagnetic labeling.

2.2.2. Immunomagnetic labeling—Following RBC lysis, the cells are resuspended in labeling buffer, counted via hemocytometer, and a desired amount of cells are aliquoted for storage before magnetic labeling. While the cell counting and aliquoting are not required, these steps assist in quality control and troubleshooting and provide cells for comparison in the subsequent staining steps. The immunomagnetic labeling protocol involves two reagents: a CD45 tetrameric antibody complex (TAC) (Stem Cell Technologies) and dextran-coated magnetic nanoparticles (Stem Cell Technologies). The TAC is bifunctional, targeting CD45 with one antibody and dextran with another.

A number of additional labeling schemes and commerically available antibody/magnetic particle combinations were previously screened, and the results published [6,7,9,11] including CD45 MicroBeads (Miltenyi Biotec), primary anti-CD45-PE (Beckman Coulter) conjugated to secondary anti-PE-MicroBeads (Miltenyi Biotec), CD45 Dynabeads (Invitrogen). The CD45 TAC was selected on the basis of its higher performance, both in percentage of $CD45^+$ cells labeled and subsequent separation purity and recovery. The high performance is thought to be due to the bifunctional contruction of the TAC combined with the two-step protocol. The anti-CD45 antibody end of the TAC, unhindered by magnetic beads, has higher affinity and specificity to $CD45^+$ cells [9,10]. To reduce the cell loss, the cells proceed to magnetic separation immediately after the incubation with magnetic nanoparticles and any unbound magnetic particles are removed during the magnetic separation step.

The detailed magnetic labeling procedure is as follows:

1. Centrifuge the cell suspension at 350*g*, 5 min. Remove the supernatant. (care should be taken to remove as much supernatant as possible to prevent dilution of the TAC solution added next).
2. Add 0.5 μl CD45 TAC per million cells, pipette thoroughly to mix.
3. Incubate 30 min, room temperature, tapping every 5 min to mix.
4. Add 1 μl nanoparticles per million cells to the labeled cell suspension. Pipette to mix.
5. Incubate 15 min, room temperature, tap every 5 min to mix.
6. Add labeling buffer to bring the total volume to 5 ml.
7. Load the sample into the magnetic sorter and proceed with separation.

2.2.3. Magnetic separation—As shown in Fig. 2A, the magnetic separation assembly requires a quadrupole magnetic separator, a syringe pump, syringes and valves. The quadrupole magnetic separator consists of a optimized design of steel and neodymium magnets which surrounds the central flow channel. Fig. 2B presents a cut away, top view of the magnet assembly, left, and simulated magnetic field gradients, right. The cell suspension after magnetic labeling is initially loaded in syringe 2. Gradually, the cell suspension flows through the channel, contained within the magnetic assembly, and into syringe 3. The magnetically labeled cells are left, magnetically attracted in a radial direction on the insider side of the outer wall of the channel; the unlabeled cells are collected by syringe 3. An optimized volumetric flow rate, balancing this magnetic attraction in the radial direction

with the shear stress created on the wall by the flowing fluid is 5 ml/min [11]. The detailed protocol is given below:

1. Pass the sorting channel (PreCelleon, Columbus, OH) through the quadrupole magnet and connect it to syringes 1 and 2.
2. Fill syringe 3 with 60 ml of labeling buffer and connect it to the bottom port of the channel.
3. With the flow directed to syringe 1, slowly fill the channel and syringe 1 by manually infusing the 60 ml labeling buffer in syringe 3, continuing until syringe 3 is empty.
4. Connect syringe 3 to the syringe pump (Harvard apparatus).
5. Turn valve 2 to allow the syringe 2 to fill with 5 ml labeling buffer, then set valve 2 to be open between syringe 2 and the sorting channel.
6. Load the sample (5 ml suspension) into the top of the syringe 2.
7. Start the syringe pump, set to refill 45 ml at 5 ml/min.
8. When the pump has finished running, close valve 3 and disconnect syringe 3 from the sorting channel.
9. Optional: If recovery of the deposited cells (those trapped in the sorting channel) is desired, syringe 1 should be refilled with approximately 35 ml labeling buffer, and a fresh syringe should be attached to valve 3. By sliding the sorting channel out of the magnet and rapidly filling the new syringe 3, the cells in the sorting channel will be swept into the new syringe 3 for and collected.
10. Centrifuge collected cell suspension at 350*g* for 5 min to pellet the cells.
11. Discard the supernatant, leaving approximately 0.5 ml labeling buffer. Resuspend the cells and count using a hemocytometer to determine number of the isolated cells.

2.2.4. Performance estimation and cell counts—While this optimized protocol attempts to reduce the variability of the process, we observe significant variability in the level of removal of RBCs and PBLs. While not necessary, we typically measure the numbers of the nucleated cells before RBC lysis, after RBC lysis, and after magnetic separation.

To obtain nucleated cell counts, the cell suspensions are added to 3% acetic acid at a ratio of 1:25 to the total volume for before-RBC-lysis and after-RBC-lysis cells, and a ratio of 1:10 for after-magnetic-separation cells. After 10 min incubation at room temperature, the cell number is counted using a Reichert Bright-Line hemacytometer (Hausser Scientific). Incubating with 3% acetic acid can dramatically reduce the background interference caused by unlysed RBCs, cell debris, or other contaminants.

As a measure of the overall depletion process performance, the total numbers of PBLs in the initial blood sample, after RBC lysis, and after magnetic separation, and the total number of cells in the blood are enumerated. With these cell counts, RBC lysis efficiency, nucleated cell log depletion and total cell log depletion are calculated as parameters for performance evaluation.

The RBC lysis efficiency is used to evaluate the PBL recovery in the RBC lysis step as Eq. (1):

$$\text{Lysis efficiency} = \frac{N_{\text{after lysis}}}{N_{\text{before lysis}}} \times 100 \quad (1)$$

where $N_{\text{before lysis}}$ and $N_{\text{after lysis}}$ are the number of nucleated cells before and after lysis, respectively.

The nucleated cell log depletion is used to evaluate the efficiency of the CD45-based magnetic negative depletion and calculated as Eq. (2):

$$\text{Nucleated cell } \log_{10}\text{depletion} = \log_{10}\left(\frac{N_{\text{before depletion}}}{N_{\text{after depletion}}}\right) \quad (2)$$

where $N_{\text{before depletion}}$ and $N_{\text{after depletion}}$ are the number of nucleated cells before and after magnetic depletion, respectively.

The total cell log depletion is used to evaluate the efficiency of the overall non-hematopoietic cell enrichment by comparing the total number of cells in the blood to the number of cells left after depletion as determined by Eq. (3):

$$\text{total cell } \log_{10}\text{ depletion} = \log_{10}\left(\frac{N_{\text{total blood}}}{N_{\text{after depletion}}}\right) \quad (3)$$

where $N_{\text{total blood}}$ is the total number of cells in the blood.

2.2.5. Cell storage—Cells are aliquoted in labeling buffer from the cell suspension before magnetic labeling and after magnetic sorting. In order to preserve cells for future analysis, the cells before or after negative magnetic enrichment are usually stored in RNAlater (Ambion) or 70% ethanol (EtOH). The cells stored in RNAlater are reserved for future nucleic acid analysis, while the cells stored in 70% EtOH with 4% paraformaldehyde (pHCHO) fixation are reserved for immunofluorescence staining.

The cells to be stored in RNAlater are first washed with 1 ml 1× PBS, then centrifuged for 5 min at 350*g*, and supernatant discarded. RNAlater is added to the cell pellet at a concentration of 100 ul per million cells. The cell are stored at 4 °C for the first 24 h and then transferred to −20 °C or −80 °C. The RNA remains intact at −20 °C for up to a year and at −80 °C for more than a year.

The cells to be stored in 70% ethanol are first washed with PBS, centrifuged, and supernatant discarded. Cells are fixed with 1 ml 4% pHCHO per million cells for 10 min. After centrifugation, the supernatant is removed and 1 ml 70% EtOH per million cells is added. The cells in 70% EtOH should be stored at −20 °C and should be stained within 2 years.

2.3. Immunofluorescence staining

2.3.1. Immunofluorescence staining reagents—Various antibodies targeting cellular proteins relevant to CTCs, including extracellular and intracellular markers, are presented in Table 1. In addition to the typical markers used to detect CTCs, i.e., DAPI, CK, CD45, and EpCAM, additional targets were selected based on reviews of current literature, interest from colleagues in clinical oncology, and relevance to cancer-related therapies.

The primary antibodies were fluorescently labeled using the secondary antibodies in Table 2. In addition, two of the most commonly-used primary antibodies were custom-conjugated directly to fluorescent dyes: CK-AF488 (clone: CK3-6H5) and CD45-AF594 (clone: HI30).

2.3.2. Immunofluorescence staining protocol—We have used the following immunofluorescence staining protocol to identify rare cells in the blood of head and neck, breast, and colon cancer patients. Without spectral deconvolution technology, routinely up to four fluorescent colors can be used simultaneously on the same slide. The staining protocol is performed on cytospins containing up to 20,000 cells. In the case of normal blood and cell line controls, the cells can be counted and a pure population added to the cytospin. For the enriched peripheral blood samples of cancer patients, the cells will include rare non-hematopoietic cells, e.g., CTCs, as well as leukocytes that were not removed during the enrichment process due to low expression of CD45. For this reason, most staining protocols include DAPI, CK, and CD45 for CTC detection as well as one additional marker for characterization.

The protocol begins by fixing the cells with 4% pHCHO, followed by permeabilization and blocking with normal serum blocking solution (NSBS). Subsequently, primary antibodies are added and incubated prior to the addition of corresponding secondary anti-bodies. Coverslips are mounted with ProLong Gold Antifade Reagent with DAPI (Life Technologies) and sealed with nail polish. The following protocol shows an example using CK-AF488, CD45-AF647, gammaH2AX-AF594 and DAPI staining [12]:

1. Fix cells with 4% pHCHO for 10 min.
2. Permeablize and block cells with NSBS for 30 min.
3. Add 100 μl diluted anti-CD45 (1:100 dilution) and anti-gammaH2AX (1:100 dilution) primary antibodies in antibody diluent (MP Biomedicals) and incubate for one hour.
4. Wash the slides three times with PBS containing 0.05% Tween-20 (PBST) for 5 min each.
5. Add 100 μl diluted goat anti-mouse AF594 (1:400 dilution) and donkey anti-rabbit AF647 (1:400 dilution) secondary antibodies in PBST and incubate for one hour in the dark.
6. Wash the slides three times with PBST for 5 min each.
7. Add 100 μl diluted CK-AF488 custom-conjugated antibody (1:100 dilution) in PBS containing 1% bovine serum albumin (BSA) and incubate for one hour in the dark.
8. Wash the slides three times with PBST for 5 min each.
9. Mount coverslip with ProLong Gold Antifade Reagent with DAPI.

The example shown above can be generalized when using unconjugated primary antibodies requiring the addition of secondary fluorescent dyes and pre-conjugated antibodies. All steps are performed at room temperature. Steps 5–8 can be skipped if all antibodies used are conjugated to fluorescent dyes, while steps 9–10 can be skipped if no pre-conjugated antibodies are used. When applying primary antibodies in the staining, different host species should be used for each to prevent cross reaction of secondary antibodies. The dilution factor varies due to different concentrations provided by antibody manufacturers, but the optimal final concentration is typically 5–10 μg/ml for primary and conjugated antibodies and 1–5 μg/ml for secondary antibodies. In all cases, the antibodies are titrated using appropriate controls to determine the optimal dilution.

2.3.3. Microscopy and imaging—Two microscope systems are used to analyze the stained slides and capture images: a Nikon Eclipse 80i epifluorescence microscope with Intesilight C-HGFi mercury vapor lamp and DS-Qi1Mc digital CCD camera, and an Olympus FV1000 spectral confocal system.

The Nikon epifluorescence microscope was equipped with the filter sets in Table 3 for detection of four-color staining with DAPI, FITC or AF488, AF555 or AF594, and AF633 or AF647.

The Olympus FV1000 spectral confocal system, located in the Campus Microscopy and Imaging Facility at The Ohio State University, is equipped with four lasers, a mercury vapor lamp, two spectral detectors, two filter-based detectors, and a transmitted DIC detector. Table 4 lists the configuration used for detection of the four-color staining.

2.4. Flow cytometry

2.4.1. Flow cytometry reagents—CD45 is typically used to exclude cells as CTCs. Even when using a CD45-based negative enrichment protocol, it is necessary to con-firm that cells of interest are CD45-negative. Beyond being necessary to identify traditional CTCs, CD45 staining of the enriched cells provides a measure of the effectiveness of the magnetic depletion. Here we used flow cytometry to investigate staining leukocytes with different anti-CD45 clones prior to, and after labeling with the anti-CD45 TAC. Investigation of potential interaction of the clones is important to ensure that CD45 staining results are not influenced by the TAC used in the separation, which could lead to false negative results for CD45 in enriched cell population. Table 5 lists the six anti-CD45 antibody from seven manufacturers used in this analysis.

2.4.2. Flow cytometry staining protocol—A two-color flow cytometry (FCM) analysis is performed on the source leukocytes for testing different CD45 clones. Three tubes are made to test each clone: (1) unstained, (2) before magnetic labeling (BML), and (3) after magnetic labeling (AML) with the anti-CD45 TAC/magnetic nanoparticles. The unstained tube is treated equivalent to the BML tube without any subsequent staining listed below.

Post-RBC-lysis source leukocytes were washed with PBS and counted. One million cells were added to each tube and the AML tubes were magnetically labeled as described above. One microliter reconstituted viability dye (LIVE/DEAD Fixable Violet Dead Cell Stain Kit, Life Technologies) was added to each tube and incubated for 30 min. After a PBS wash, the corresponding clone of anti-CD45 antibody was added to the AML and BML tubes and incubated for 30 min. All cells were washed with PBS again and re-suspended in 300 μl of PBS before FCM analysis.

2.4.3. Flow cytometer—All experiments were conducted on a BD FACS LSR II instrument, located in the Analytical Cytometry Shared Resource at The Ohio State University, using the FACS Diva software for data acquisition and analysis. The LSR II instrument was calibrated using cytometer setup and tracking (CST) and DNAQC beads in the CST module in the Diva software. Automatic compensation was performed per Diva protocols using unstained and single-color controls. At least 10,000 events were recorded per sample for analysis. FlowJo 10.0.6 (Tree Star) is used to set the population gates and analyze the data.

3. Results and discussion

3.1. Immunomagnetic separation performance

The immunomagnetic separation step discussed uses a highly optimized magnetic and fluidic assembly which imposes on the cell suspension a magnetic gradient on the order of 300 T/m and hydrodynamic forces which significantly reduce non-specific binding of cells to the surfaces in contact with the cells within the assembly. The magnetic depletion is accomplished as the cell suspension flows through an open annular channel, which is surrounded by the quadrupole magnet, and the magnetically labeled cells are retained on outer, inner channel wall by the very high magnetic gradient. This magnetic pressure created to hold the magnetically labeled cells against the outer, inner wall of the annulus is sufficient to allow a relatively rapid fluid flow through the annulus, with corresponding shear stress which reduce non-specific binding. The unlabeled cells flow through the system and are collected for further analysis. It is noted that care was taken to maintain this shear stress below levels known to cause cell damage.

To measure the performance of the negative immunomagnetic separation, the results of 120 breast cancer patient samples are presented here. Both the total cell count and nucleated cell count of the initial sample are recorded, as well as nucleated cell counts after both RBC lysis and magnetic depletion. Eq. (1)–(3) were used to determine the performance of the RBC lysis as well as the magnetic depletion. Eq. (2) was used to calculate the nucleated cell log depletion, useful for determining the specific performance of the quadrupole magnetic separator, and Eq. (3) was used to calculate the total cell log depletion, which includes the performance of the RBC lysis. For the breast cancer patient samples in this study, the average nucleated cell log depletion is 2.56, while the average total cell log depletion is 5.63. Therefore, on average, only 0.28% of nucleated cells remain after depletion, while 0.0002% of the total blood cells remain.

Since the immunomagnetic separation described here is based on depletion of hematopoietic cells, it is important to consider not only the number of cells removed at each step, but also the number of cells of interest that are retained. For the RBC lysis, the objective is to remove RBCs, but it is also important to retain as many nucleated cells as possible. A measure of the retained cell performance can be calculated from Eq. (1), the lysis efficiency. The average lysis efficiency for the breast cancer patient set analyzed here is 77%. For the magnetic depletion, the analogous performance measure is CTC recovery. However, in patient samples it is impossible to know with certainty the initial number of CTCs. An estimation of this performance, based on spiking studies, is presented in the next section.

3.2. Recovery of spiked cancer cell lines

A common challenge with respect to evaluating the performance or rare cell separation/isolation technologies, such as the separation/recovery of circulating tumor cells, is the proper criteria/model systems to use. Unfortunately, in many cases, the number and type of rare cells in actual human samples is unknown. While spiking studies are commonly used, such as spiking cancer cells (from a cancer cell line) into normal blood, this assumes that the cancer cell line is a true representation of CTCs. Never-the-less, cancer cells lines spiked into normal human blood is a first step in developing performance standards.

Fig. 3 is a log–log plot of the number of cancer cells recovered versus the number of cancer cells initially spiked into normal blood (the type of cancer cells is listed in the legend). This data was obtained over a six-year period, with four different cancer cell lines and five different researchers. All numbers are reported as the number of cancer cells per ml of blood equivalent (a value of 6×10^6 nucleated cells per ml of blood was assumed, which is consistent with the data observed with cancer patient blood samples). The lower plot is an

enlarged view of a portion of the plot above to show better detail in the range of 10–100 cells per ml blood equivalent. The dark, solid line corresponds to a 100% recovery. Linear regression lines through the origin are presented for two cell lines: MCF7 and SCC4. Regression analysis of the MCF7 data (light, solid line) produced a slope of 0.39 (95% CI: 0.37–0.42) and correlation coefficient (R^2) of 0.99. Analysis of the SCC4 data (dash line) produced a slope of 0.84 (95% CI: 0.83–0.85) and an R^2 of 1.00. The other cell lines, F-01 and Detroit 562, were tested at a single spiking level, but linearity studies were not performed.

It is important to note the relatively high level of performance observed with the SCC4, F-01, and Detroit 562 cell lines (~80% recovery) compared to the poor recovery of MCF-7 (~45%). A number of speculations can be made for the poor performance of MCF-7, including significant clumping which compromised accurate cell counts. However, such variation with respect to different cell lines underscores the care that must be taken in interpreting and comparing spiking studies.

3.3. Comparison of CD45 antibody clones using flow cytometry

The flow data from all tubes are first gated on forward scatter and side scatter to remove cell debris and select singlet events, followed by gating on the viability dye based on the results from the unstained tube to select only viable cells. All remaining events are used for further quantitative analysis of the CD45 clones. Fig. 4A presents FCM histograms of the six anti-CD45 antibody clones from seven manufacturers tested in the CD45 flow analysis. The unstained tubes were used to set the threshold between negative and positive events in each case. Based on these results, a majority of AML events from the clones of F10-89-4_NB, 5B1, 2D1 and HI100 are negative, while the BML events of these clones are positive. The AML curve of clone J.33 is partially shifted but remains positive. The AML curves of clone HI30 and F10-89-4_AbD are only slightly shifted.

To further assist in the interpretations of these histograms, after multiple repeats, Fig. 4B presents the percentage of cells considered positive for each of the antibody clones. A good clone, which is suitable for use in analysis applications to identify CD45 positive cells in the enriched fraction of the separation, should not only stain majority of leukocytes, but have little change in the percentage of positive cells between the BML and AML groups.

The TAC is clone MEM 28, and is reactive with all isoforms of CD45. In this experiment we identify other clones which exhibit no change in staining prior to or after magnetic labeling, and given that CD45 is a pan-leukocyte marker, we would expect greater than 97% of the cells should be positive [10]. Significant variation between clones can be observed with respect to the fraction of the cells that are positive for CD45. Although all of the clones are effective for staining the BML fraction, in the AML samples the incompatible clones fail to provide an adequate number of positive cells, and in the case of F10-84-9_NB give no positivity at all.

These results indicate that for our specific study, only clones J.33, HI30, and F10-89-4_AbD are acceptable as both a general PBL stain as well as not being inhibited when the cell is previously labeled with the CD45 TAC (clone MEM 28). By identifying these compatible clones, potential staining inconsistencies leading to false negative results for CD45 on the isolated cell fraction can be reduced.

3.4. Multi-marker staining of enriched peripheral blood samples from cancer patients

3.4.1. Nuclei and CD45—When analyzing slides, it is necessary to first identify cells and exclude debris and other artifacts of staining. In immunofluorescence, this identification is

commonly based on the presence of nuclei. DAPI is a reliable staining reagent for cell nuclei. Cytospins mounted with ProLong Gold Antifade Reagent with DAPI and stored at 4 °C, the DAPI fluorescence will remain for up to two years.

Since the immunomagnetic separation described here is based on removal of hematopoietic cells, including leukocytes, it is necessary to identify and exclude any of these cells that remain after enrichment. Being a common marker for nucleated hematopoietic cells, CD45 is an important judge in the traditional definition of CTC. Based on the results of the FCM analysis above, clone HI30 was selected and used in most of the following immunofluorescence images. The demonstration of both DAPI and CD45 staining are shown in the figures of tumor-related markers in the following sections.

3.4.2. Epithelial markers—Although not all epithelial cells in human blood necessarily originate from a tumor site [13], the positive expression of epithelial markers is used as an important criterion to identify CTCs. In most circulating tumor cell research, CK and EpCAM are used as two typical epithelial markers. CK is a family of 29 proteins shaping the intermediate filament in the cytoskeleton of epithelial cells. Breast cancer usually expresses CK 7, 8, 18, 19 and selectively expresses CK 5, 6 in some specific subtypes, such as basal-like [14]. For this reason, anti-CK 8, 18, 19 antibody is applied in the CK staining studies.

The second commonly used epithelial marker is EpCAM. Unlike CK, which is intracellular, EpCAM is a cell surface marker which can be targeted with an antibody for positive selection [1]. However, evidence from multiple sources are beginning to indicate that not all circulating tumor cells express EpCAM [1,15]. Some aberrant expression of EpCAM, such as up-regulated, down-regulated or de novo expression are found in epithelial tumor cells [16]. These aberrant expressions make the total expression of EpCAM vary depending on the type of cancer and the progression of carcinoma. Fig. 5 presents the result of four color staining and microscopy analysis, of a triple negative breast cancer patient sample enriched in the manner presented in this report along with control samples. The patient sample demonstrates that rare non-hematopoietic cells exist that are not always consistent with both CK and EpCAM markers present on the same cell.

3.4.3. Mesenchymal markers—Epithelial-to-mesenchymal transition (EMT) has been reported to exhibit dynamic changes within the progression of breast cancer and chemotherapy. Research is beginning to emerge that EMT can take place in solid tumors and that both mesenchymal and epithelial CTCs can be present [17,18]. Immunofluorescence staining with mesenchymal markers is therefore necessary to identify these CTCs which do not conform to the traditional definition.

Vimentin is an intermediate filament protein, which is a feature of mesenchymal cells. In the cells undergoing EMT, without changing the other markers of EMT, vimentin induces the alteration of cell shape, adhesion and motility [19]. It is widely used as a marker to study epithelial and mesenchymal protein co-expression on CTCs in the phenotypic state of EMT [19–23].

Cadherin can mediate cell adhesion with the regulation of calcium ion. Within the cadherin family, N-cadherin (N-CAD) and E-cadherin (E-CAD) are major regulators of adhesion and integrity of epithelial cells, and a shift from E-CAD to N-CAD is associated with EMT [21].

Variations in CK/Vimentin expression level are observed between different cells from the same patient. Fig. 6A shows an enriched cell from patient blood with immunofluorescence staining of CK and vimentin. The patient cell appears CK positive, CD45 negative, VIM weakly positive, thus can be regarded under EMT process. With the addition of N-CAD,

Fig. 6B presents some patient cells that are positive on CK, N-CAD and vimentin. The staining setting of CK, N-CAD and vimentin can be used in further EMT research to identify the cells undergo EMT and to analyze the EMT progress on tumor cells. If additional markers are needed, either N-CAD or vimentin can be taken out from the setting in Fig. 6B and vacate the channel for the additional markers. Fig. 7 shows a patient cell under EMT (CK+ and VIM+), as well as EGFR positive, which is a cell surface marker from the human epidermal growth factor receptor family that induces cell differentiation and proliferation and is activated by 7 growth factors.

3.4.4. Other markers of emerging interest—Beyond the typically reported epithelial and mesenchymal markers discussed above, a range of other markers have elicited interest in the rare cell analysis field. Cells positive on some of these markers are found in the enriched cells from cancer patient blood specimens.

These markers include HER2, in the same family as EGFR (Fig. 8), G-protein coupled surface receptor in Hedgehog (Hh) pathway, Smo (Fig. 9), transcription factors mediating the Hh pathway, Gli1 (Fig. 10), and a cellular response to DNA damage marker, gammaH2AX [12].

3.5. Antibody choice for immunofluorescence staining

With the same antibody clone, in terms of binding specificity, antibodies pre-conjugated to fluorescent dyes performs better than combinations of unconjugated primary antibody and secondary fluorescent dyes, due to decreased non-specific binding. It is also important to design an experiment with only a single primary antibody from a given host species. Significant non-specific binding will appear if two unconjugated primary antibodies from the same species of host are added onto the same immunofluorescence staining slide even with additional blocking steps. However, the presence of the non-specific binding can be decreased if antibodies conjugated to fluorescent dyes are applied in the staining. For uncommon antibodies, some protein labeling kits can successfully be applied as reported by other researchers [24], and are preferred to minimize non-specific binding.

3.6. Device and fluorescent dye choice for immunofluorescence staining

The emission spectra for common fluorescent dyes are typically quite broad, with a bandwidth of 50 nm or more. Therefore, using two fluorescence dyes that have close emission peaks (usually within about 50 nm) can lead to spectral overlap and thus false positivity (Fig. 11A). For this reason, few researches show five-or-more-color immunofluorescence staining on the same cells. An acceptable combination of four fluorescent dyes that span the visible spectrum can be AF405 or DAPI, FITC or AF488, AF555 or AF594, and AF633 or AF647. The orange-red fluorescent dyes, including AF546, AF555, and AF594 (or Texas Red), cannot be differentiated using standard filter-based methods [25].

Mercury vapor bulbs are a commonly used light source for epifluorescence microscopy. However, the spectrum of a mercury vapor bulb is not uniform, rather it has several distinct peaks. To obtain the best results, the antibody with weaker binding to target cells should be matched with the fluorescent dye whose excitation peak overlaps one of the primary peaks of the mercury vapor bulb. On the other hand, the antibodies with stronger binding to target cells can use a fluorescent dye whose excitation peak is at a relatively low part of the spectrum of the mercury vapor bulb, to eliminate the interference towards neighboring fluorescence channels. In our four-color staining, AF405, AF555, and AF594 are better choices for antibodies with weaker binding, since their excitation peaks partially overlap with the emission peaks of mercury bulb. However, the AF488 and AF647 are suitable for

antibody with stronger binding to target cells. (Fig. 11B) If all antibodies applied in a single staining have weak binding to the target cells, it would be better to use a more powerful and focused light source, such as lasers.

4. Conclusion

Negative selection for CTCs is superior to positive selection with respect to not being limited by an initial selection on a single (or potentially multiple) cell surface marker(s). We have developed a robust, rare cell enrichment methodology which allows further analysis for a wide range of cell markers. An optimized, four-color immunofluorescence staining protocol demonstrates the heterogeneity of these enriched samples, beyond the traditional CTC markers of CK and EpCAM. The use of these additional markers including the mesenchymal markers, vimentin and N-cadherin, the epidermal growth factor receptors, EGFR and HER2, markers related to the Hedgehog pathway, Gli1 and Smo, and a DNA damage indicator, gammaH2AX, demonstrated this heterogeneity. However, these range of different markers underscores the need for multiparameter analysis that can target more than four markers at a time.

References

1. Van de Stolpe A, Pantel K, et al. Cancer Res. 2011; 71(18):5955–5960. [PubMed: 21896640]
2. Cristofanilli M, Budd G, et al. N Eng J Med. 2004; 351:781–791.
3. De Bono JS, Scher HI, et al. Clin Cancer Res. 2008; 14:6302–6309. [PubMed: 18829513]
4. Stott S, Hsu CH, et al. PNAS. 2010; 107(43):18392–18397. [PubMed: 20930119]
5. Yu M, Stott S, et al. J Cell Biol. 2011; 192(3):373–382. [PubMed: 21300848]
6. Lara O, Tong X, et al. Exp Hematol. 2004; 32:891–904. [PubMed: 15504544]
7. Yang L, Lang J, et al. Biotech Bioeng. 2009; 102:521–534.
8. Miller B, Chalmers J. Methods Cell Biol. 2012; 112:183–192.
9. Zhang H, Williams PS, Zborowski M, Chalmers JJ. Biotechnol Bioeng. 2006; 95:812–829. [PubMed: 16937410]
10. Ralph S, Thomas S, et al. EMBO J. 1987; 6:1251–1257. [PubMed: 2956090]
11. Tong X, Yang L, et al. Cytometry B Clin Cytometry. 2007; 72(5):310–323.
12. Garcia-Villa A, Balasubramanian P, et al. Front Oncol. 2012; 2:128. [PubMed: 23112954]
13. Goeminne JC, Guillaume T, et al. Ann Oncol. 2000; 11(7):785–792. [PubMed: 10997804]
14. Karantza V. Oncogene. 2011; 30(2):127–138. [PubMed: 20890307]
15. Balasubramanian P, Lang JC, et al. PLoS One. 2012; 7:7.
16. Trzpis M, McLaughlin P, et al. Am J Pathol. 2007; 171(2):386–395. [PubMed: 17600130]
17. Yu M, Bardia A, et al. Science. 2013; 339(6119):580–584. [PubMed: 23372014]
18. Powell A, Talasaz A, Zhang H, et al. PLoS One. 2012; 7:5.
19. Mendez M, Kojima S, et al. FASEB J. 2010; 24(6):1838–1851. [PubMed: 20097873]
20. Armstrong A, Marengo M, et al. Mol Cancer Res. 2011; 9(8):997–1007. [PubMed: 21665936]
21. Sarrió D, Rodriguez-Pinilla SM, et al. Cancer Res. 2008; 68(4):989–997. [PubMed: 18281472]
22. Mikolajczyk, Stephen D.; Millar, Lisa S.; Tsinberg, Pavel, et al. Detection of EpCAM-Negative and Cytokeratin-Negative Circulating Tumor Cells in Peripheral Blood. J Oncol. 2011; 68:252361. http://dx.doi.org/10.1155/2011/25236. [PubMed: 21577258]
23. Sieuwerts A, Kraan J, et al. J Natl Cancer Inst. 2009; 101(1):61–66. [PubMed: 19116383]
24. Karadag A, Ogbureke K, et al. J Natl Cancer Inst. 2004; 96–12:956–965.
25. Schieker M, Pautke C, et al. J Anat. 2004; 204(2):133–139. [PubMed: 15032920]

Appendix A. Buffer formulations

Table A.1

Selected buffer and reagent formulas.

Component	Amount	Concentration
Lysis buffer (10×)		
Distilled water	250 ml	–
Ammonium chloride (#A649, Fisher Scientific)	20.1 g	1.5 M
Ethylenediamine tetraacetic acid, EDTA (#BP121, Fisher)	074 g	1 mM
Sodium bicarbonate (#BP328, Fisher)	2.1 g	140 mM
Labeling buffer (1×)		
PBS, 1X (#21-040-CM, Corning Cellgro)	1 L	–
EDTA	74 g	3 mM
Bovine serum albumin (#BP1605, Fisher)	5 g	0.5%
4% paraformaldehyde		
Distilled water	35 ml	–
PBS, 10X (BP399, Fisher)	5 ml	1×
Paraformaldehyde, 20% (#15723, EMS)	10 ml	4%
Normal serum blocking solution (NSBS)		
PBS, 1×	50 ml	–
Normal goat serum (#005-000-121, Jackson ImmunoResearch)	1 ml	2
Normal donkey serum (#017-000-121, Jackson IR)	1 ml	2
Bovine serum albumin	0.5 g	1
Gelatin (#G7041, Sigma)	50 mg	0.10
Triton X-100 (#BP151, Fisher)	50 ul	0.10
Tween 20 (#BP337, Fisher)	25 ul	0.05%

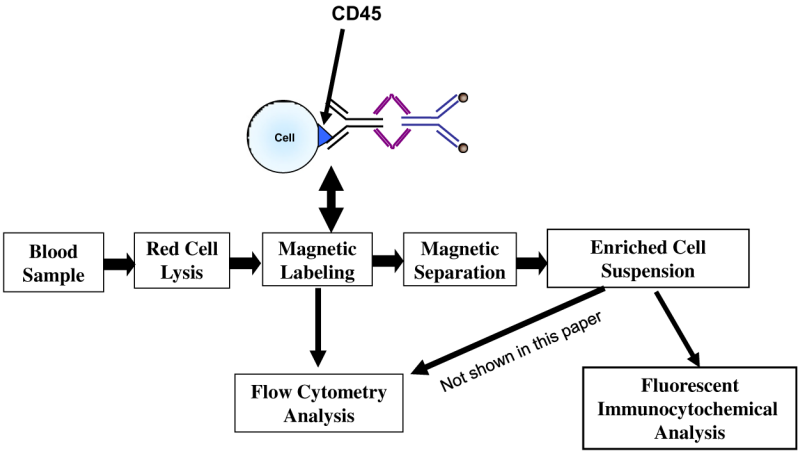

Fig. 1.
Flow chart of the CD45-based negative depletion and analysis pathway.

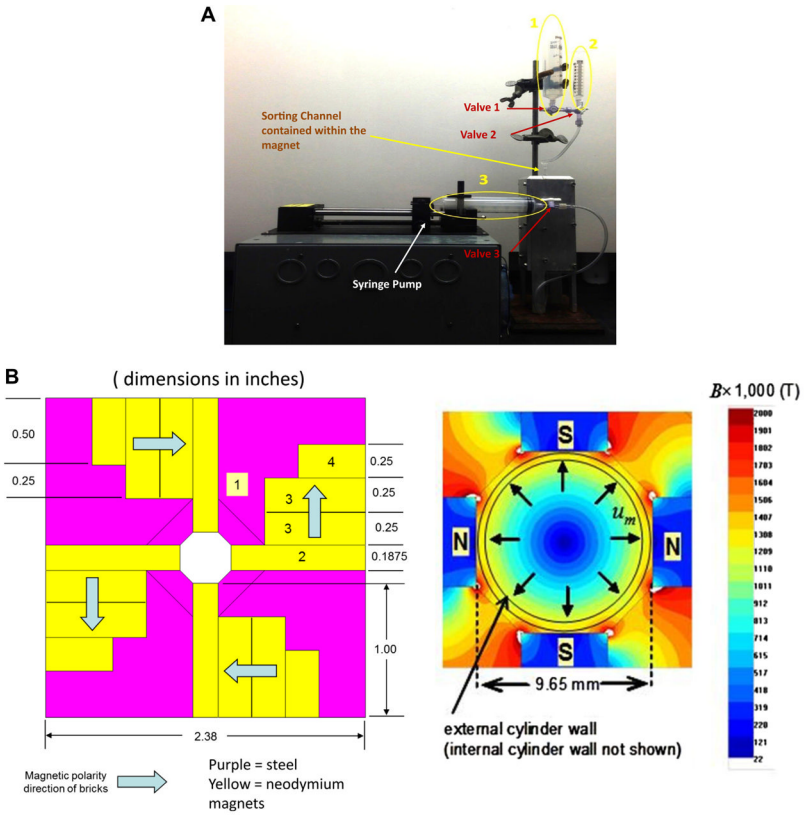

Fig. 2.

(A) Schematic diagram of the complete setup of the quadrupole magnetic sorter. The valve and syringe labels correspond to the protocol in Section 2.2.3. (B) Top, cut away, view of the quadruple magnet assembly, LHS, and contour plot of magnetic field strength in the quadrupole magnet with annular channel dimensions superimposed, RHS.

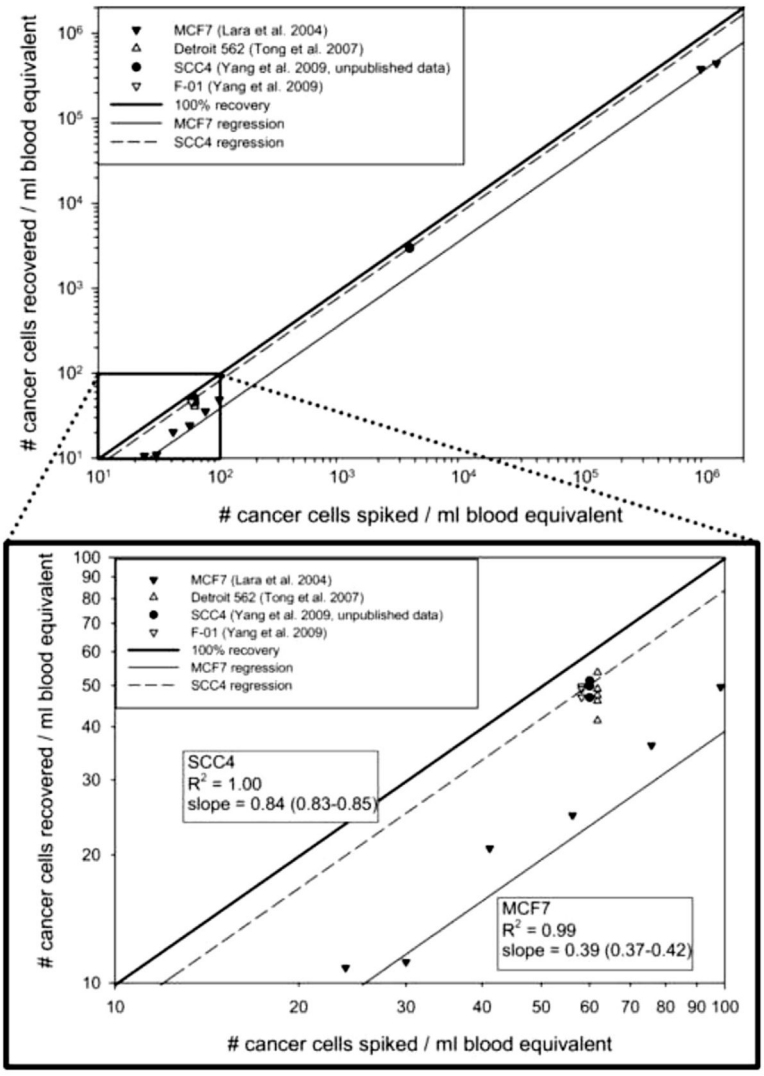

Fig. 3.

Log–log plot showing the results of the cancer cell line spiking tests. Bottom, enlarged view of a portion of the top plot to show better detail in the range of 10–100 cells per ml blood equivalent. Four cell lines are tested. The thick line corresponds to 100% recovery. The thin line is a linear regression through the origin of the MCF7 cell line data and the dash line is a regression of the SCC4 cell line data. Note that the F-01 and Detroit 562 symbols are shifted slightly for clarity (All calculations are based on the original data).

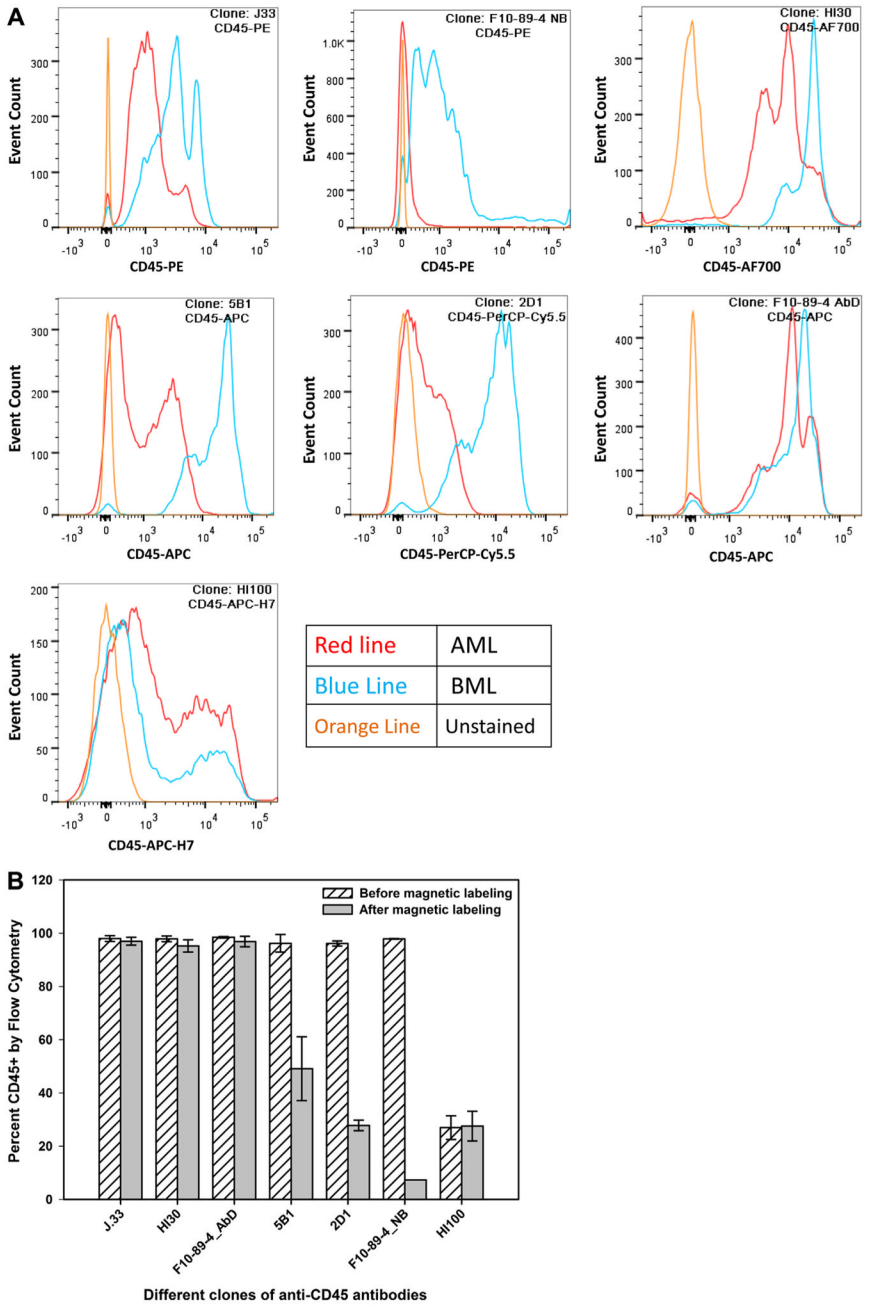

Fig. 4.

(A) Histograms of various anti-CD45 antibody clones, based on FCM analysis of peripheral blood leukocytes, both before (blue) and after (red) immunomagnetic labeling with anti-CD45-TAC/magnetic nanoparticles. Unstained controls are shown in orange. (B) Bar graph comparing the percentage of cells positive for each clone of anti-CD45 antibody, based on FCM analysis, both before and after immunomagnetic labeling.

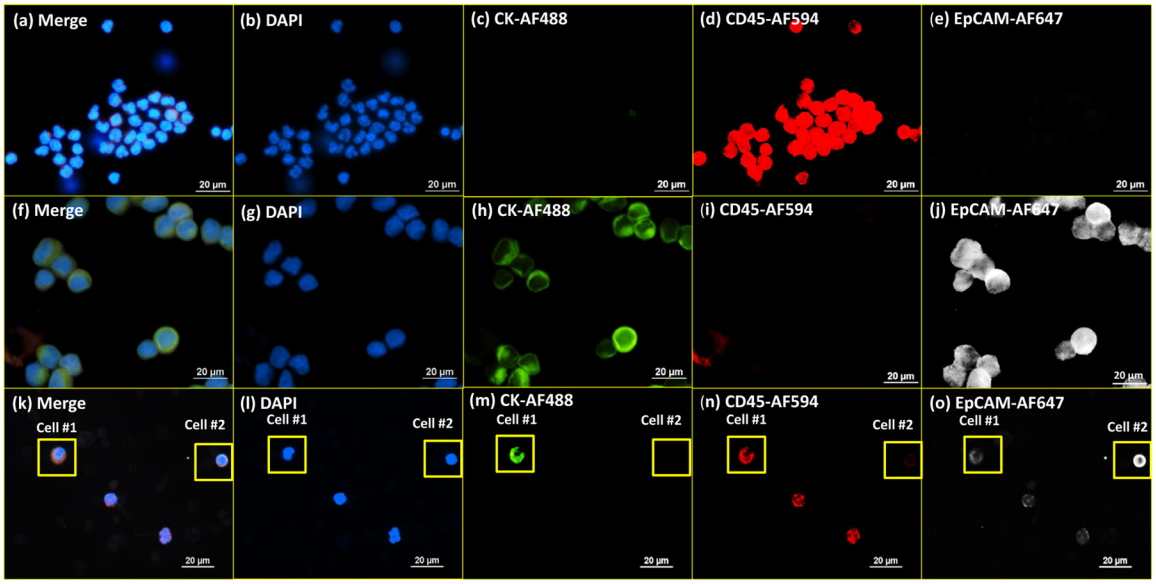

Fig. 5.

Four-color images of a cytospin stained for DAPI, CK, CD45, and EpCAM of (a–e) leukocytes, (f–j) breast cancer cell line MCF7, and (k–o) an enriched peripheral blood sample from a metastatic breast cancer patient. The first column is a combined image of the four colors and the remaining columns (left to right) are DAPI, anti-CK-AF488, anti-CD45-AF594, and anti-EpCAM-AF647, respectively. For the patient sample, Cell #1 is $CK^{+}CD45^{+}EpCAM^{-}$ and Cell #2 is $CK^{-}CD45^{+}EpCAM^{+}$.

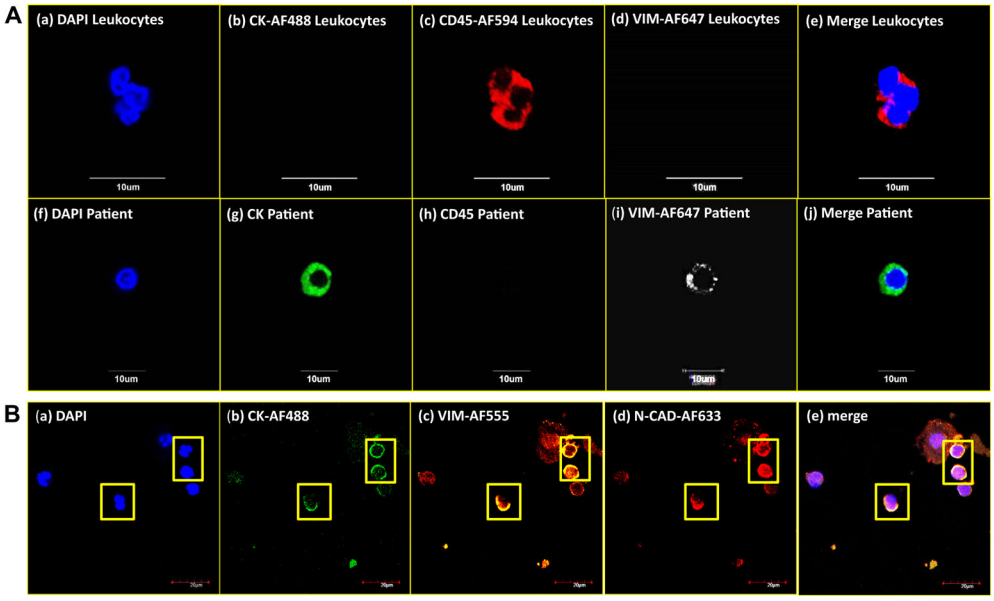

Fig. 6.

(A) Four-color images of a cytospin stained for DAPI, CK, CD45, and VIM of (a–e) leukocytes, and (f–j) an enriched peripheral blood sample from a cancer patient. The columns (left to right) are DAPI, anti-CK-AF488, anti-CD45-AF594, anti-VIM-AF647, and a combined image of the four colors, respectively. (B) Four-color image of a cytospin stained for DAPI, CK, Vim, and N-CAD of an enriched peripheral blood sample from a cancer patient. (a) DAPI; (b) anti-CK-AF488; (c) anti-Vim-AF555; (d) anti-N-CAD-AF633 and (e) combined image of the four colors. The cells in the yellow boxes are $CK^{+}Vim^{+}N\text{-}CAD^{+}$. Reproduced with permission from Balasubramanian et al. [15].

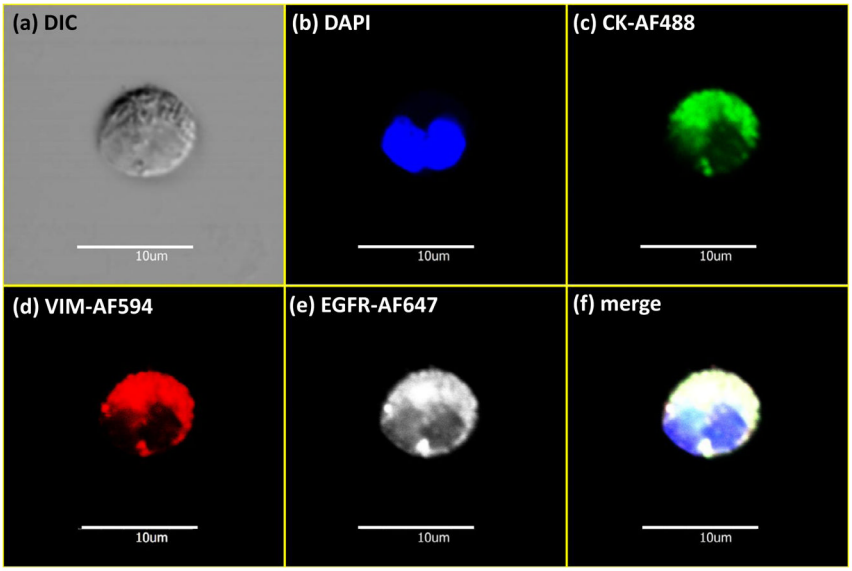

Fig. 7.

Four-color image of a cytospin stained for DAPI, CK, Vim, and EGFR of an enriched peripheral blood sample from a metastatic breast cancer patient. (a) DIC; (b) DAPI; (c) anti-CK-AF488; (d) anti-Vim-AF594; (e) anti-EGFR-AF647 and (f) combined image of (b–e). The cell shown in the figure is $CK^{+}Vim^{+}EGFR^{+}$.

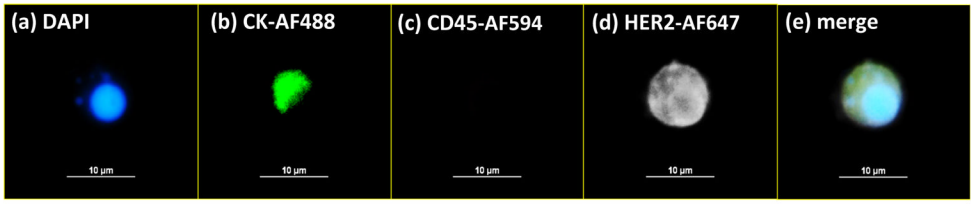

Fig. 8.

Four-color image of a cytospin stained for DAPI, CK, CD45, and HER2 of an enriched peripheral blood sample from a metastatic breast cancer patient. (a) DAPI; (b) anti-CK-AF488; (c) anti-CD45-AF594; (d) anti-HER2-AF647 and (e) combined image of the four colors. The cell in this figure is $CK^{+}CD45^{-}HER2^{+}$, a HER2-expressing CTC.

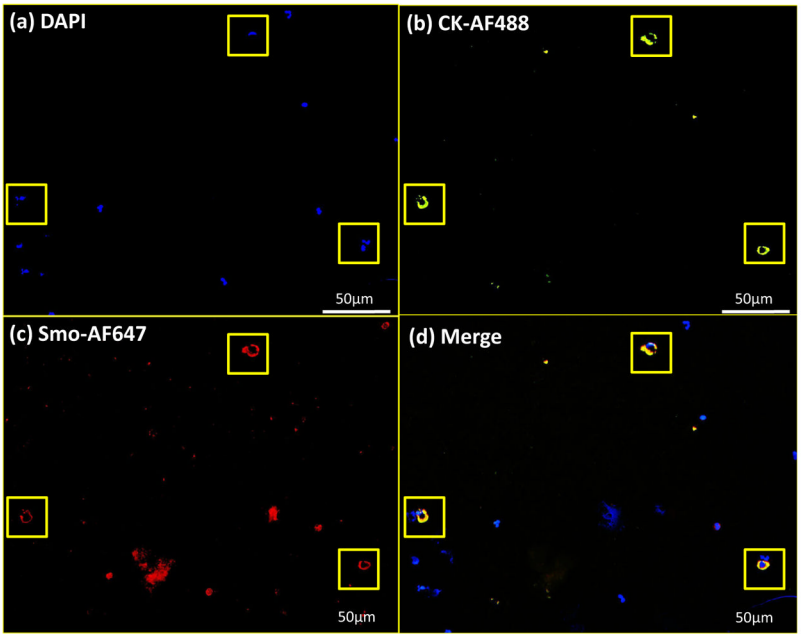

Fig. 9.

Three-color image of a cytospin stained for DAPI, CK, and Smo of an enriched peripheral blood sample from a cancer patient. (a) DAPI; (b) anti-CK-AF488; (c) anti-Smo-AF647 and (d) combined image of the three colors. The three cells in the yellow boxes are $CK^{+}Smo^{+}$.

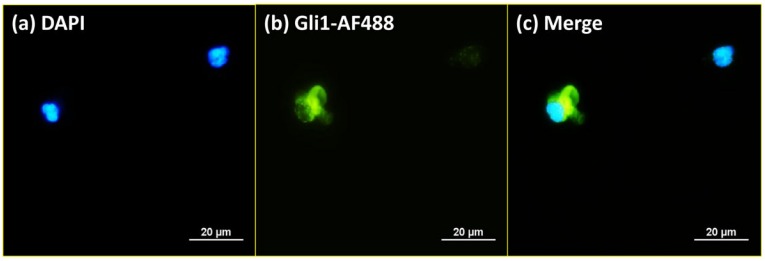

Fig. 10.

Two-color image of a cytospin stained for DAPI and Gli1 of an enriched peripheral blood sample from a cancer patient. (a) DAPI; (b) anti-Gli1-AF488 and (c) combined image of the two colors. The cell on the left is $Gli1^{+}$ and the cell on the right is $Gli1^{-}$.

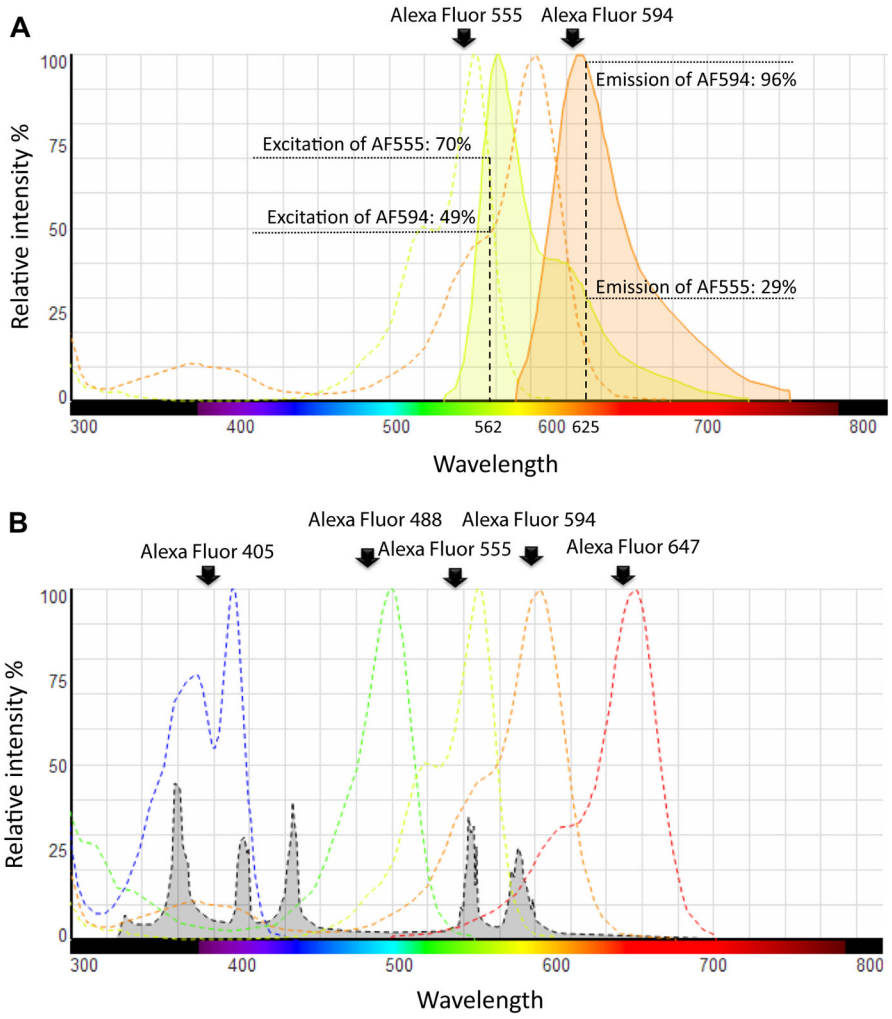

Fig. 11.

(A) Excitation and emission spectra of AF555 and AF594, showing relative excitation and emission intensities when excited at 562 nm and detected at 625 nm, respectively. Note the significant overlap between the dyes, which prevents accurate simultaneous detection when using traditional microscopic techniques. Modified based on the original plot obtained from the Fluorescence SpectraViewer (Life Technologies). (B) Excitation and emission spectra of selected Alexa Fluor dyes: AF405, AF488, AF555, AF594 and AF647. The gray spectrum represents the emission from a typical mercury vapor lamp. Modified based on the original plot obtained from the Fluorescence SpectraViewer (Life Technologies).

Table 1

Table of primary antibodies.

Target	Clone	Host species	Manufacturer
Nucleus (DAPI)	–	–	Life Technologies
CK 8, 18, 19	CK3-6H5	Mouse	Miltenyi Biotec
CD45	HI30	Mouse	BD Biosciences
CD45	(Polyclonal)	Rabbit	Abcam
EpCAM	(Polyclonal)	Rabbit	Abcam
EGFR	(Polyclonal)	Rabbit	Abcam
HER2	29D8	Rabbit	Cell Signaling Tech.
N-CAD	(Polyclonal)	Rabbit	Abcam
Vimentin	(Polyclonal)	Goat	Abcam
Gli1	D-1	Mouse	Santa Cruz Biotech.
Smo	(Polyclonal)	Rabbit	Abcam
GammaH2AX	JBW301	Mouse	Millipore

Table 2

List of secondary antibodies.

Fluorescent dye	Target (IgG)	Host species
AF488	Anti-mouse	Goat
AF555	Anti-goat	Donkey
AF594	Anti-mouse	Goat
AF633	Anti-rabbit	Donkey
AF647	Anti-goat	Donkey
AF647	Anti-rabbit	Donkey

Table 3

Filter sets in the Nikon epifluorescence microscope.

Filter set	Excitation (BP)	Dichroic	Emission	Fluorescent dyes
Blue	377/50	409	447/60 BP	DAPI
Green	475/50	506	536/40 BP	FITC or AF488
Orange	535/50	565	572 LP	AF555
Red	562/40	593	625/40 BP	AF594
Far red	620/60	660	700/75 BP	AF633 or AF647

Table 4

Configuration of lasers and detectors in the Olympus FV1000 spectral confocal microscope.

Excitation laser	Emission	Fluorescent dyes
405 nm (Diode)	425/50 BP spectral	DAPI
488 nm (Argon/2)	525/50 BP spectral	FITC or AF488
543 nm (HeNe1)	560 LP filter	AF555 or AF594
633 nm (HeNe2)	650 LP filter	AF633 or AF647

Table 5

List of CD45 antibody clones, fluoroprobes, and manufacturers tested.

Clone	Fluorescent dye	Manufacturer
J.33	PE	Beckman Coulter
F10-89-4_NB	PE	Novus Biologicals
F10-89-4_AbD	APC	AbD Serotec
HI30	AF700	Biolegend
5B1	APC	Miltenyi Biotec
2D1	PerCP-Cy5.5	BD Biosciences
HI100 (CD45RA)	APC-H7	BD Biosciences