

resulted in a wider distribution of capture efficiencies. This is attributed to the increased time required to carry out the separation. At 0.5 ml/hr, the sample requires approximately 1 hour to pass through the sifter, during which the cells in the inlet tube undergo significant sedimentation to the bottom of the tube before flowing through the sifter.

[0049] The capture yields for these experiments, and the linear relationship between capture yield and flow rate were extremely encouraging, especially given the small patterned area of the sifter ($\sim 20 \text{ mm}^2$), which is on the order of 50 times smaller than most microdevices devices geared towards capturing CTCs from whole blood.

[0050] In addition to 40 μm sifters, separations were performed on H1650 spiked into whole blood using 50 μm square pores, the bowtie shaped pores discussed above, as well as 20 μm circular and square pores. Capture experiments using 50 μm pores showed no observable change in capture efficiency. Separation with the bowtie structure resulted in lower capture efficiencies (50% at 5 ml/hr), but cells were captured at predictable locations between the pole tips. This capture behavior can be advantageous when the imaging and enumeration of cells is performed automatically by an optical scanning system.

[0051] In the case of 20 μm pore sizes, it was found that the H1650 were captured from whole blood with efficiencies of 80% regardless of flow rate. After it was observed that the H1650 were captured with the same efficiency in the absence of an applied external field, and also without magnetic labeling, it became apparent that the H1650 were captured based on their comparably larger sizes and reduced deformability for the 20 μm pore sizes.

Experiment 2

Fluorescent Staining of Bare H1650 Cells

[0052] The second significant hurdle to overcome is to fluorescently label the tumor cells in a background of whole blood, in a selective manner for identification and enumeration. Experiments were thus performed on bare H1650 spiked into whole blood, without any pre-fluorescent or pre-magnetic labeling, to mimic the conditions of separating and enumerating CTCs from a clinical sample.

[0053] Differentiating a captured tumor cell from a WBC requires multi-color imaging of at least two fluorophores, one to indicate a positive expression of a surface antigen or intracellular protein specific to the tumor cell, and another to indicate positive expression of a surface antigen specific to a WBC. In preliminary experiments, captured cells were stained with biotin-PE (red) and anti-CD45-FITC (green). Tumor cells should fluoresce in the red, since they are positive for EpCAM, and hence should display the streptavidin-coated ferrofluid which is stained red by the biotin-PE. Also, a tumor cell should be absent in measurements of green fluorescence, or, negative for CD45. Likewise, WBCs should appear green, since they are positive for CD45 and are stained with anti-CD45-FITC, while they should be absent in measurements of red fluorescence (negative for EpCAM).

[0054] Preliminary experiments staining captured H1650 with anti-EpCAM-PE revealed weak fluorescence, likely due to the saturation of EpCAM binding sites by the anti-EpCAM-biotin selection antibody. As a result, the biotin-PE was used to amplify the red fluorescence above background. In

actual clinical samples, cell permeation and staining with cytokeratin, an intracellular protein specific to CTCs, will likely be used.

[0055] Human EpCAM+ Cancer Cells PlusCollect™ labeling kits and the corresponding MagCollect™ Ferrofluid were purchased from R&D Systems, Inc. The PlusCollect™ labeling kit includes a cocktail of biotinylated anti-EpCAM antibodies (concentration proprietary), a “PlusCollect™” buffer solution of proprietary composition for labeling the cells, and streptavidin-functionalized magnetic nanoparticles (MNP). The magnetic properties and manufacturer specifications of the ferrofluid are as follows: average diameter 145 nm, composition Fe_3O_4 (80% w/w)/Polymer Matrix, saturation field ~ 2000 Oe, magnetization 313 emu/cc, concentration 4×10^{11} particles/mg iron ($\sim 10^{11}$ particles/ml), and binding capacity $\sim 15,000$ small biotinylated molecules/particle ($\sim 5,000$ large biotinylated molecules/particle).

[0056] In this work, the following magnetic labeling protocol was employed (Magnetic Labeling Protocol 1). H1650 pre-fluorescently labeled with Green CellTracker™ dye were added to 0.5 mL of whole blood at concentrations of approximately 100 H1650 cells/ml. 20 μL of the selection antibody was added, in addition to 100 μL of 10% Pluronic-F-68 surfactant. The sample volume was then increased to 1 mL by the addition of PlusCollect™ buffer and incubated for 15 minutes at 4° C. The sample volume was then brought to 15 mL with PlusCollect™ buffer, centrifuged at 300G for 10 minutes, and the supernatant was removed. 40 μL of MagCollect™ Ferrofluid was added to the cell pellet, in addition to 100 μL of 10% Pluronic-F-68 surfactant. The volume was then brought to 1 mL by the addition of PlusCollect™ buffer and incubated for 20 minutes at 4° C. The sample was then washed by the addition of PlusCollect™ buffer and centrifugation, and the resulting cell pellet was then resuspended in 1 mL of PlusCollect™ buffer. The cell suspension was then processed with the sifter containing 40 μm square pores at 5 ml/hr, yielding an average capture efficiency of $53.5 \pm 4.9\%$ over five separations.

[0057] To demonstrate feasibility, bare H1650 cells were spiked into whole blood and labeled according to Magnetic Labeling Protocol 1, described above. Following magnetic labeling, 10 μL of the biotin-PE fluorophore (mg/ml) and 20 μL of anti-CD45-FITC were added to the sample, which was then incubated for 30 minutes before washing. The blood sample was then processed with the sifter at 5 ml/hr.

[0058] Merged images of the sifter surface were inspected. In these images, the white light, red fluorescence, and green fluorescence images are merged. Red fluorescent H1650 cells are observed in the sifter pores. The H1650 cells appear on the top or bottom side of the pore, at the edge perpendicular to the direction of the externally applied field. Also observed at the edges of the pores and in regions connecting pores are deposits of unbound ferrofluid, which is also stained red during the biotin-PE labeling step. WBCs are not found anywhere on the surface, indicating they pass through the sifter uncaptured or are removed during the washing step.

[0059] To enumerate CTCs from an actual patient sample, in which CTCs may not be as uniform in size, shape, and antigen expression as a well-defined cultured cell-line, more sophisticated fluorescent labeling and imaging can be used. A typical protocol involves staining the nuclei of both CTCs and WBCs, in addition to one positive antigen of CTCs and one positive antigen of WBCs. To evaluate 3-color imaging on the sifter surface, two combinations of dyes were tested on bare