

Magnetophoretic Mobilities Correlate to Antibody Binding Capacities

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Methods: A methodology and a mathematical theory have been developed, which allow quantitation of the expression levels of cellular surface antigens using immunomagnetic labels and cell tracking velocimetry (CTV) technology.

Results: Quantum Simply Cellular (QSC) microbeads were immunomagnetically labeled with anti-CD2 fluorescein isothiocyanate (FITC) antibodies and anti-FITC MACS paramagnetic nanoparticles. Magnetophoretic mobility has been defined as the magnetically induced velocity of the labeled cell or microbead divided by the magnetophoretic driving force, proportional to the magnetic energy density gradient.

Discussion: Using computer imaging and processing technology, the mobility measurements were accomplished by microscopically recording and calculating the velocity of immunomagnetically labeled QSC microbeads

in a nearly constant magnetic energy gradient. A calibration curve correlating the measured magnetophoretic mobility of the immunomagnetically labeled microbeads to their antibody binding capacities (ABC) has been obtained.

Conclusion: The results, in agreement with theory, indicate a linear relationship between magnetophoretic mobility and ABC for microbeads with less than 30,000 ABC. The mathematical relationships and QSC standardization curve obtained allow determination of the number of surface antigens on similarly immunomagnetically labeled cells. *Cytometry* 40:307-315, 2000.

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Key terms: antigen density; quantitative flow cytometry; antibody binding capacity; magnetic cell separation; magnetophoretic mobility; particle tracking velocimetry

The ability to separate cells has important applications in the diagnosis and treatment of disease. A current application attracting interest is the separation of hematopoietic progenitor cells from human umbilical cord blood, normal bone marrow, and peripheral blood. These cells are able to reconstitute hematopoiesis in humans and may be used for the treatment of patients having undergone chemotherapy (1,2). Other medical applications for cell separation include the isolation of cancer cells from blood for analysis and an early diagnosis (3) and the isolation of fetal cells from blood for DNA testing.

Current commercially available cell separation technologies include fluorescent-activated cell sorting (FACS), Mini MACS, Dynabeads, MPC (particulate magnetic beads), CELLector flasks (panning technique), and CellPro (avidin-coated beads in a column; 4). Of these cell separation systems, FACS is the only method that uses a flow mode of separation, as opposed to batch mode. Although batch devices are relatively simple, they have limited throughput volumes and provide little control over the separation process. FACS, however, is expensive and still has relatively slow separation rates.

Nonbatch, high-throughput quadrupole and dipole immunomagnetic cell sorting devices are currently under

development in our laboratories (5,6). These devices can be used for large-scale isolation of cell populations. Two distinct advantages are that these magnetic flow sorters are significantly less expensive and potentially orders of magnitude faster than FACS. In addition, magnetic flow sorting does not induce the high cellular shear stresses that are present in FACS separation.

In continuous immunomagnetic cell separators, the design and operation are highly dependent on the degree to which a cell is immunomagnetically labeled. This "degree of immunomagnetic labeling" can be quantitated by obtaining the magnetic susceptibility and the magnetophoretic mobility of the immunomagnetically labeled cell (7,8). A technique to determine the magnetic susceptibility of an immunomagnetically labeled cell (or particle), on a cell-by-cell-basis, has been developed (9). This technique involves videotaping the movement of immunomagneti-

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cally labeled cells through a medium of known viscosity and magnetic susceptibility in a well-defined magnetic energy density gradient. The velocity of each cell along with its location within the magnetic energy gradient is recorded. From this information, the magnetic susceptibility of each cell is obtained. The technique, referred to as cell tracking velocimetry (CTV), now includes semi-automated computer video imaging and tracking analysis (7,10). The CTV magnet has a region of nearly constant magnetic energy gradient, which greatly simplifies the analysis (8).

An analogy can be drawn between the CTV system and fluorescent-activated cell scanning in flow cytometry (FCM). In the CTV system, the measured parameter is the induced velocity of the immunomagnetically labeled cell when placed within the magnetic energy gradient. In an FCM system, the analogous measured parameter is the fluorescence intensity (FI) of an immunofluorescently labeled cell or particle when excited by a laser beam of the appropriate wavelength. However, the induced velocity (or magnetophoretic mobility) in the CTV system is reported on an absolute scale, millimeter per second (or $\text{mm}^3/\text{T}\cdot\text{A}\cdot\text{s}$, millimeter cubed per tesla-ampere-second). In FCM, the FI signal is an amplified and relative value that requires significant standardization and control (11). Also, due to this absolute nature of magnetic and CTV technology, equations can be developed that model the movement of an immunomagnetically labeled cell or particle within the magnetic energy gradient.

One parameter of interest governing the measured magnetic or fluorescent signal is the antibody binding capacity (ABC). ABC is a term that provides quantitative information about the number of antibodies binding to the surface molecules on individual cells. To conveniently obtain quantitative information about cell surface molecule expression levels, microbead standards that bind antibodies in a well-calibrated manner are required. Quantum Simply Cellular microbeads (Flow Cytometry Standards Corporation, San Juan, Puerto Rico) are one example of such a microbead standard used for FCM. These microbeads have varying capacities to bind mouse monoclonal antibodies (12). When fluorochrome-conjugated mouse monoclonal antibodies bind to these specific receptors, the microbead's FI from FCM is directly proportional to the number of antigen molecules on that microbead or cell labeled with similar antibodies. FCM analysis of immunofluorescently labeled cells, along with immunofluorescently labeled microbead standards correlating FI with ABC, allows quantitation of the cells' surface molecule expression levels (11). Similarly, when paramagnetic material is bound to the antigen sites on this microbead standard, the induced mobility will be directly proportional to the number of surface antigens. Therefore, we hypothesize that magnetophoretic mobility is also directly proportional to ABC.

MATHEMATICAL MODEL OF MAGNETOPHORESIS

The equations presented here are modified from previously published work and will provide a new, more thor-

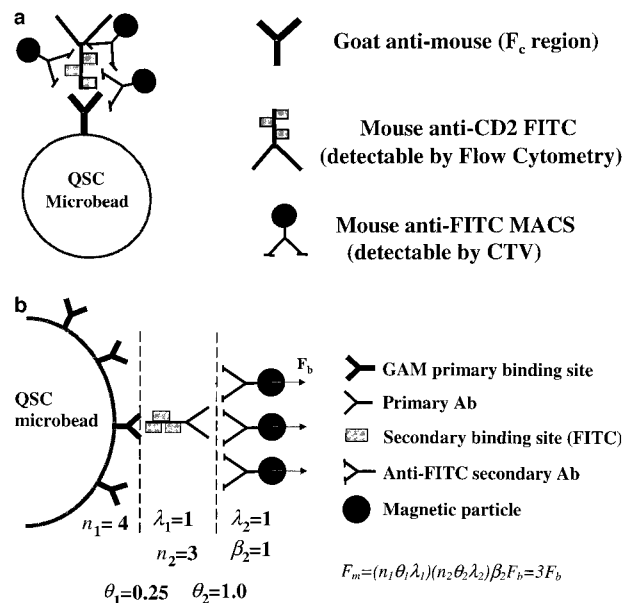


FIG. 1. **a:** Diagram of immunomagnetic labeling of Quantum Simply Cellular (QSC) microbeads. **b:** Diagram of amplification of immunomagnetic particle binding using three magnetic microbeads per one antigen site as an example (see Equation 1).

ough attempt to investigate and quantify all of the related parameters that contribute to the magnetically induced movement of an immunomagnetically labeled particle (8). For a paramagnetically labeled cell or microbead, the forces impacting its movement through a liquid suspension are magnetic (F_m), buoyancy (F_{bou}), gravity (F_g), and drag (F_d) forces. Using a two-step labeling protocol (Figs. 1a,b), the paramagnetic force acting on an immunomagnetically labeled cell or microbead can be represented as:

$$F_m = (n_1\theta_1\lambda_1)(n_2\theta_2\lambda_2)\beta_2F_b \quad (1)$$

where subscripts 1 and 2 refer to the primary and secondary labeling step, respectively, n_j is the number of antigen molecule sites per cell or microbead, including specific and nonspecific antigen sites ($n_s + n_{ns}$), and θ_j is the fraction of antigen molecules on the particle surface bound by the primary antibody. The parameter, λ_j , represents the valence of the primary antibody binding. For example, the CH4 molecule is known to exhibit bivalent antibody binding, meaning that one antibody binds two CD4 antigen molecules, thus the value for λ_j would be 1/2 (13).

The combined term $n_j\theta_j\lambda_j$ is equivalent to the ABC of a cell population (12). ABC is a measure of the number of primary antibodies binding to a cell or microbead. This value includes not only the number of antigen molecules per cell, but also variables such as valence of antibody binding, steric hindrance, binding affinities, and nonspecific binding. Thus we have:

$$ABC = n_1\theta_1\lambda_1 \quad (2)$$

The same sequence of parameters is then repeated for the binding of the secondary antibody to sites on the primary antibody. In this case, n_2 is the number of binding sites on the primary antibody recognized by the secondary antibody. For example, if the secondary antibody target is a fluorescein isothiocyanate (FITC), n_2 will be the number of FITC molecules conjugated to the primary antibody. This value is often referred to as the fluorescein-to-protein ratio (F/P) of the antibody reagent. θ_2 is the fraction of binding sites on the primary antibodies that are bound by the secondary antibodies and λ_2 represents the valence of the secondary antibody binding (i.e., the number of secondary antibodies that bind per one FITC molecule). n_3 is the number of magnetic nanoparticles conjugated to the secondary antibody. The parameters $n_2\theta_2\lambda_2n_3$ may be combined into one overall term, β . In this case, β represents the number of magnetic nanoparticles bound to each primary antibody on a cell or microbead. The lumped term, βABC , describes the number of magnetic nanoparticles bound to each cell or microbead, and is therefore referred to as the “magnetic particle binding capacity” of a cell or microbead.

F_b is the magnetic force acting on one paramagnetic nanoparticle in the direction of the magnetic energy gradient and is described by:

$$F_b = \frac{1}{2\mu_0} \Delta\chi V_m \nabla B^2 \quad (3)$$

where μ_0 is the magnetic permeability of free space, $\Delta\chi$ is the difference in magnetic susceptibility between the magnetic material, χ_b , and the surrounding medium, χ_f . V_m is the volume of paramagnetic material per paramagnetic nanoparticle and B is the magnetic flux density.

As long as the Reynolds number is less than 0.1, one can assume that drag forces follow Stokes' equation, thus F_d is defined as:

$$F_d = 3\pi\eta D_c \nu_c = f\nu_c \quad (4)$$

where ν_c is the velocity of the cell moving through the fluid, D_c is the diameter of the cell or microbead, η is the viscosity of the fluid, and f is the friction coefficient of the moving cell or microbead:

$$f = 3\pi\eta D_c \quad (5)$$

For the previous equations, the following assumptions have been made. It has been assumed that the number of surface molecules expressed on the cell, n_1 , is independent of the cell size, or D_c . This assumption is justified by the practice of FCM analysis, in which the cells may be independently gated by their size. Typically, for a given size gating, there is considerable variation in cell FI (proportional to antigen expression), indicating that there are

significant differences in the antigen numbers expressed on cells that are essentially the same size.

However, if, in fact, the cell has an internal regulatory feedback control on the number of surface molecules it expresses per cell membrane surface area, then the number of antigen molecules on a cell would be related to the diameter of the cell. Preliminary work investigating the theoretical effect of cellular size on the velocity (mobility) and cell separation assumed this dependence, using the term “antigen density” (14). Because this cellular internal feedback control of antigen numbers per membrane surface area is not a consistently true phenomenon, we now choose to treat the variables n_1 and D_c separately, thus the model may be applied whether antigen expression is a function of or is independent of cell size.

Second, n_2 , θ_2 , λ_2 , n_3 , and thus β , as well as $\Delta\chi$ and V_m , are all independent of ABC. This suggests that the total paramagnetic force acting per immunomagnetically labeled surface molecule remains the same for all labeled surface molecules. This also requires that the same volume of total paramagnetic material is bound to each antibody and thus to each targeted surface molecule of interest, and that the binding affinities of antibodies to antigens are independent of ABC. The values of n_2 , θ_2 , λ_2 , n_3 , $\Delta\chi$, and V_m may actually vary, but these variations cancel out when averaged over tens of thousands of surface binding sites. The overall effect from each of these parameters will remain the same from cell to cell or from particle to particle. Third, drag forces arising from the magnetically induced velocity of a cell are the only flow effects on the labeled cell or microbead. Fourth, due to the rather large size of a cell, thermal and Brownian motion are assumed to be negligible.

The force balance on the cell or microbead can be written as:

$$mass * acceleration = F_m + F_d + F_g + F_{bou} \quad (6)$$

For micrometer-sized particles, the nonstationary term on the left hand side of the equation is several orders of magnitude smaller than the magnetic and drag forces of fast moving particles, thus, we set the left side of the equation equal to zero (9). Also, because the magnetic energy gradient in the experimental system is perpendicular to gravity, we are only interested in the horizontal movement of the cell or microbead through the fluid. This reduces the force balance equation to:

$$0 = F_m - F_d \quad (7)$$

Substituting Equations 1, 2, and 4 into Equation 7 and noting that magnetic and drag forces are in opposite directions, we obtain a relationship for the magnetic energy gradient-induced velocity, ν_c , of the moving paramagnetized cell or microbead:

$$\nu_c = \frac{(n_1\theta_1\lambda_1)(n_2\theta_2\lambda_2)\beta_2|F_b|}{3\pi D_c \eta} = \frac{ABC\beta|F_b|}{f} \quad (8)$$

where the straight brackets, $| \cdot |$, denote magnitude of a vector. The magnetophoretic driving force, S_m , is proportional to the magnetic energy gradient and is defined as:

$$S_m = \frac{|\nabla B^2|}{2\mu_0} \quad (9)$$

and magnetophoretic mobility, m , is defined as:

$$m = \frac{v_c}{S_m} \quad (10)$$

Substituting Equation 3 into 8 and then Equations 8 and 9 into 10, we obtain an equation for the magnetophoretic mobility, a “normalized” parameter analogous to electrophoretic mobility, of an immunomagnetically labeled cell or microbead:

$$m = \frac{\Delta\chi V_m}{3\pi D_c \eta} \beta_{ABC} = \frac{k}{f} \beta_{ABC} \quad (11)$$

where $k = \Delta\chi V_m$ is a constant representing the magnetic property of a single magnetic nanoparticle.

The above equation describes the parameters contributing to the magnetophoretic mobility of a single immunomagnetically labeled cell or microbead. Within a population of immunomagnetically labeled cells, each individual cell will differ slightly from other cells in size and ABC, resulting in a measurable distribution of mobilities for a given cell population. Ideally, we would like to independently measure the two parameters, cell size and ABC, for each single cell. Unfortunately, advancements in CTV technology still limit simultaneous measurements of size and mobility. Thus, for now, we will deal with the mean size, ABC, and mobility of each cellular distribution.

Again, the lumped term, β_{ABC} , in Equation 11 represents the magnetic particle binding capacity of a cell or microbead. Thus, Equation 11 concludes that the magnetophoretic mobility of an immunomagnetically labeled cell or microbead is directly proportional to the number of magnetic nanoparticles binding to the cell or microbead and that the magnetophoretic mobility is inversely proportional to the cell or microbead’s friction coefficient. We will now investigate the predicted proportionality between m and ABC .

EXPERIMENTAL METHODS

Quantum Simply Cellular Microbeads

Quantum Simply Cellular microbeads are uniform, 8.5- μm polystyrene microbeads with calibrated numbers of goat anti-mouse antibodies (GAM) bound to their surfaces. Individual sets of these microbeads are coated with four distinct populations of GAM antibodies that bind the Fc region of IgG1, IgG2a, and IgG2b isotypes of mouse monoclonal antibodies and one blank microbead population for controls. Quantum Simply Cellular microbeads were custom shipped in individually packaged vials of uniform antibody binding populations (lot A03366), hav-

ing median ABC values of approximately 0, 4,500, 16,000, 52,000, and 210,000. Each vial of these microbeads contains 2 million microbeads per milliliter. Simply Cellular microbeads are single-population, GAM-coated microbeads having a median ABC of approximately 66,000.

Primary Labeling of Microbeads by Immunofluorescent Antibody

Approximately 1.5 million of these microbeads (750 μl) were placed in microcentrifuge tubes and adjusted to 1.5 million microbeads per milliliter with phosphate-buffered saline (PBS). Saturating amounts (15.5 μl) of primary mouse (isotype IgG2a) anti-CD2 FITC antibodies (CD2 Leu-5b, Becton Dickinson, San Jose, CA, lot 00199, F/P = 4.66) were added to the tubes and incubated for 1 h at 7°C. These microbeads were then washed with PBS.

Secondary Labeling of Microbeads by Immunomagnetic Antibody

For CTV analysis, the immunofluorescently labeled microbeads were resuspended in 500 μl of PBS and then given saturating amounts (50 μl) of secondary mouse (isotype IgG1) MACS anti-FITC antibodies (Miltenyi Biotec, Auburn, CA, lot NE8163) and incubated for 30 min at 7°C. These microbeads were then washed twice with PBS and resuspended in 750 μl of PBS for CTV analysis. Figure 1a depicts the standard microbead with both primary and secondary antibody labels.

Antibody Saturation Studies

Experiments were conducted to ensure that the concentration of antibody reagent for immunolabeling microbeads was sufficient to saturate the available antigen binding sites. To ensure this, the microbead population with the greatest number of antigen binding sites (receptors) was incubated with different concentrations of FITC and paramagnetically conjugated antibody reagents following the protocol above. Because the primary antibody was an antibody-FITC conjugate, FCM was used for the saturation study of the primary antibody. CTV was used for saturation studies of the secondary, paramagnetically conjugated, antibody. Optimum amounts were then used for labeling all ABC microbead populations.

CTV Apparatus

The CTV image analysis system was designed to measure the velocity of the paramagnetically labeled cells or other similarly sized particles in a well-defined magnetic energy gradient (Fig. 2). The microbead sample was pumped with a Harvard PhD 2000 Programmable Syringe Pump (Holliston, MA), fitted with a 1-ml syringe, in the negative z-direction through flexible tubing into a 1-mm inner diameter (ID) square glass channel. An inverted microscope with a 5 \times objective was focused on the microbeads in the glass channel at the appropriate region of constant force. Light was supplied to the microscope by a Fiber Lite (Dolan-Jenner, Lawrence, MA) fiber optic light source with a fiber optic cable. The movement of the cells or microbeads in the magnetic energy gradient was

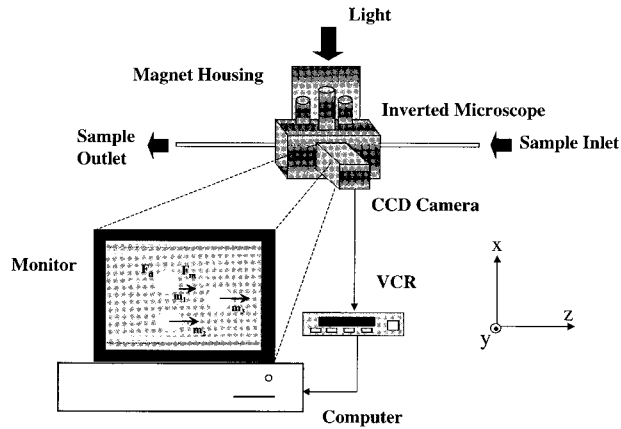


FIG. 2. Diagram of CTV experimental setup.

videotaped with a 30-Hz Cohu (San Diego, CA) CCD 4915 camera and a Sony SVO-95000MD video recorder. A more thorough discussion of the apparatus can be found in previous publications (7,8,10).

CTV Analysis

Analog VCR images from videotapes were converted into 624×450 pixel images in which each pixel was assigned a gray level ranging from 0 (black) to 255 (white or brightest) with a MuTech image board (Mutech, Billerica, MA) and M-Vision 1000 Sequence software (Mutech). This software allows the user to adjust gain and offset settings to improve image quality before recording and saving images from the videotape or directly from the camera, and converting them into pixel (digital) form. The software also allows the user to choose how many total frames of images to save as well as how many frames to skip before saving the next image. This frame-skipping feature is an important tool that can sometimes significantly reduce noise in the final velocity data.

Execution of proprietary Borland C++ programs, which identify the moving particles and calculate their velocities, allows the velocities for each cell/microbead tracked to be converted into magnetophoretic mobilities using mathematical models. More details are provided in separate publications (7,8,10).

RESULTS

Histograms of the five ABC populations of the immunomagnetically labeled Quantum Simply Cellular microbeads are seen in Figure 3a, which is a composite of five separate experiments with each QSC microbead population. The x-axis represents the magnetophoretic mobility values of each immunomagnetically labeled microbead population. The y-axis represents the fraction of beads with that specific magnetophoretic mobility. Note that the histograms for each ABC population are distinct and that the mean magnetophoretic mobility for each ABC microbead population increases with increasing ABC values. Histograms of the control studies of Quantum Simply Cellular mi-

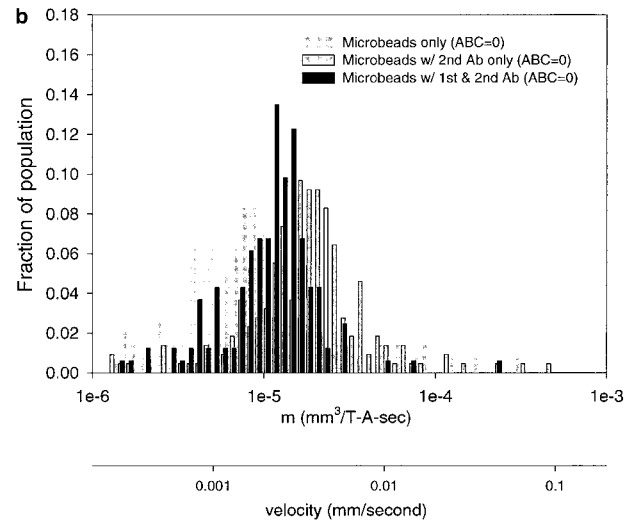
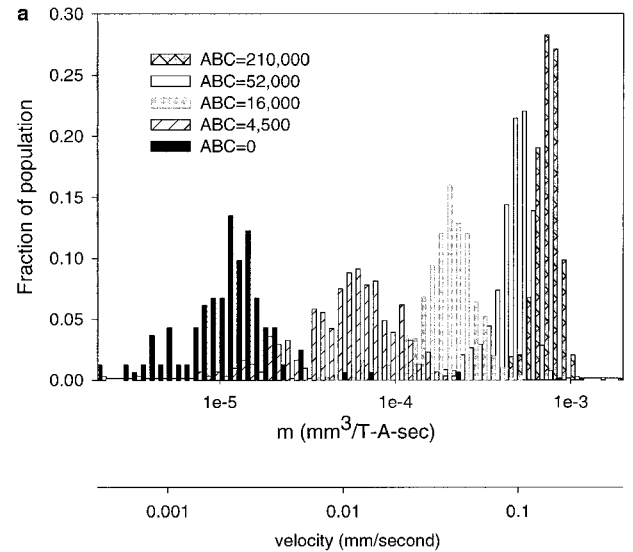


FIG. 3. **a:** Histograms of the magnetophoretic mobilities of the immunomagnetically labeled Quantum Simply Cellular microbead populations. **b:** Histograms of the magnetophoretic mobilities of the controls for Quantum Simply Cellular microbeads.

crobeads with different combinations of antibody labeling are presented in Figure 3b. These control studies were conducted on the microbead population with ABC equal to zero.

Table 1 lists the means, medians, modes, SDs, and coefficients of variation (CV) of the measured magnetophoretic mobilities, as well as the number of microbeads analyzed in each experiment. These rather large SDs for the mobilities for each of the Quantum Simply Cellular microbead populations represent the inherent distribution in the numbers of the surface molecules per microbead as well as additional variation due to microbead sizes, immunolabeling methods, and CTV. The reported SDs and CVs are common to both FCM and CTV analysis methods. (The CVs for Quantum Simply Cellular mi-

Table 1
Listing of the Means, Medians, Modes, SD, CV, and the Sample Size (N) of the Magnetized Quantum Simply Cellular (QSC) Bead Magnetophoretic Mobilities

QSC population	m (mm ³ /T-A-s)			SD	CV (%)	N
	Mean	Median	Mode			
ABC = 0, no antibody	1.58E-05	6.95E-06	7.50E-06	3.51E-05	221	145
ABC = 0, 2nd antibody	3.25E-05	1.86E-05	1.68E-05	1.01E-04	310	217
ABC = 0	1.37E-05	1.19E-05	1.19E-05	1.89E-05	140	163
ABC = 4,500	6.01E-05	5.54E-05	5.96E-05	3.54E-05	59	307
ABC = 16,000	2.12E-04	2.09E-04	2.11E-04	7.63E-05	36	498
ABC = 52,000	4.67E-04	4.78E-04	5.31E-04	1.50E-04	32	1,057
ABC = 210,000	7.60E-04	7.67E-04	7.50E-04	1.70E-04	22	782

Table 2
Results of a Reproducibility Study of the Magnetized Simply Cellular Beads on 2 Different Days Using Two Different Lots of the Magnetizing Reagent (anti-FITC MACS microbead)*

Simply cellular	m (mm ³ /T-A-s)			SD	CV (%)	N
	Mean	Median	Mode			
Lot 8480-day 1	2.77E-04	2.89E-04	2.99E-04	8.79E-05	32	472
Lot 8480-day 1	3.22E-04	3.28E-04	3.35E-04	7.98E-05	25	515
Lot 8480-day 2	3.50E-04	3.50E-04	3.76E-04	1.11E-04	32	875
Lot 8163-day 1	2.67E-04	2.61E-04	2.66E-04	1.18E-04	44	436

*The nomenclature is the same as that in Table 1.

crobeads using FCM analysis range from 39% to 13%.) We expect a slightly larger CV from CTV than from FCM because CTV required a two-step antibody-labeling procedure, whereas FCM only required a one-step antibody-labeling procedure. The lowest CTV signal intensities, however, report significantly larger CVs than the analogous CVs from FCM signals. This is the result of the decreasing mean mobility rather than an increasing SD and is explained by the definition of CV (SD divided by the mean). In CTV, the mean mobilities are nearly zero (approximately 0.001 mm/s) for the slowest or nonmoving particles. Thus, we expect the CV for the slowest mobilities to be very high. We do not see a large value for the CV of weakly fluorescent cells in FCM because of the inherent nonzero autofluorescence of the microbeads.

Statistical analysis on Quantum Simply Cellular microbeads was conducted using SigmaStat. The results of the Tukey pairwise multiple comparison test indicate that the differences in the means of each ABC microbead treatment group (Fig. 3a) are all statistically significant ($P < 0.05$). The statistical significance in the differences in mean mobilities obtained from the control populations (Fig. 3b) was not found to be significant ($P < 0.05$). In order to be confident that the mean and median values are accurate, many particles from one population need to be analyzed. CTV usually analyzes up to about 1,000 particles for each sample population. In this way, the means of each sample population have statistical significance.

The mean magnetophoretic mobility of each ABC microbead population was plotted against its corresponding median ABC for that population (Fig. 4). Bars around each mean mobility data point represent the 99% confidence

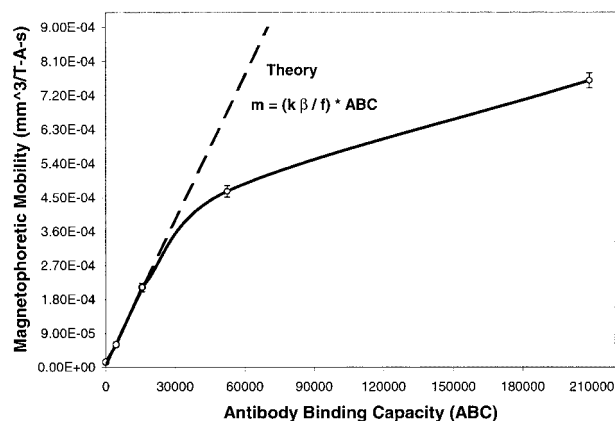


Fig. 4. Plot of the magnetophoretic mobilities versus the given ABC of the immunomagnetically labeled Quantum Simply Cellular microbeads. The linear portion of the experimental curve (solid line) is described by equation $m = 1.27 \times 10^{-8} + 9.01 \times 10^{-6}$.

intervals for each population mean. The confidence intervals are narrow, even with rather large SDs, because of the large number of mobility data analyzed, or large sample size (15).

Note that at higher ABC values, the plot in Figure 4 exhibits saturation-like behavior. It will be shown that steric hindrance issues become important for microbeads with high numbers of antigen binding sites labeled with these paramagnetic nanoparticles. The calibration plot is linear and follows theory for the lower ABC populations, as expected from Equation 11. The slope of the linear portion of the calibration plot is:

$$\frac{k\beta}{f} = \frac{\Delta\chi V_m \beta}{3\pi D_c \eta} = 1.27 \times 10^{-8} [mm^3/T - A - s] \quad (12)$$

where:

$$\beta = n_2 \theta_2 \lambda_2 \beta_2 \quad (13)$$

The combination of theory and direct observation of the Quantum Simply Cellular microbead movement in the magnetic field provides important information about the interaction between the Quantum Simply Cellular microbead and the bound magnetic nanoparticles. First, it can be assumed that the finite size of the magnetic nanoparticles influences the antibody-nanoparticle binding kinetics by a steric hindrance mechanism, thus causing this deviation from linearity at high ABC values. As the ABC value increases, more magnetic nanoparticles bind to the Quantum Simply Cellular microbead. Eventually, the space available for these magnetic nanoparticles to bind will be depleted. The average diameter of the Quantum Simply Cellular microbead is 8.5 μm , surface area of $2.27 \times 10^{-10} \text{m}^2$. Product information from Miltenyi Biotec indicates that the average diameter of the MACS nanoparticle is approximately 50 nm, cross-sectional area of $1.96 \times 10^{-15} \text{m}^2$. The maximum number of nanospheres covering the surface of the Quantum Simply Cellular microbead is calculated to be 105,000. This calculation assumes that both types of spheres behave like rigid bodies and that the magnetic nanospheres form a densely packed hexagonal lattice on the surface of the Quantum Simply Cellular microbead (90.7% coverage). Also, the nanospheres form only one layer on the Quantum Simply Cellular microbead and the curvature of the Quantum Simply Cellular microbead may be neglected. Looking at Figure 4 and remembering that the lumped term, βABC , describes the number of magnetic nanoparticles bound to the cell or microbead, conclusions may be made regarding the value of β . The deviation from linearity occurs at about 27,000 ABC. If one assumes that the point at which the deviation from linearity occurs is when the maximum number of nanoparticles has covered the surface of the Quantum Simply Cellular microbead, then $\beta = 4$.

The calculated value of $\beta = 4$ allows us to estimate some of the parameters entering into Equation 13. Product information from Becton Dickinson indicates that the F/P ratio for the specific lot of anti-CD2 FITC antibodies used in these experiments is equal to 4.66. In the context of this work, F/P is equivalent to n_2 , therefore, $n_2 = F/P = 4.66$. Product information from Miltenyi Biotec indicates that $n_3 = 1$ for MACS antibody-nanoparticles. Entering the values of β , n_2 , and n_3 into Equation 13, we obtain that $\theta_2 \lambda_2 = 0.89$. In other words, our results indicate that, on average, as expected, less than one secondary antibody is binding per epitope on the primary antibody.

Second, the linear portion of the dependence of m on ABC allows one to make inferences about the magnetic

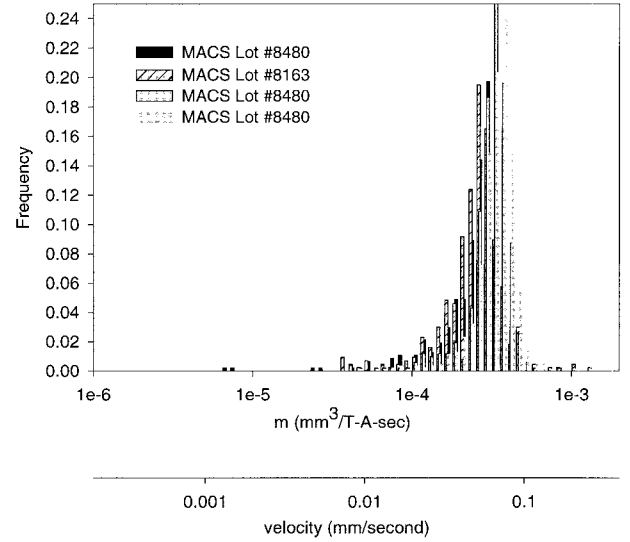


FIG. 5. Histograms of immunomagnetically labeled Simply Cellular microbeads with identical and different lots of MACS paramagnetic antibody labels.

properties of the magnetic labeling particles, the MACS nanoparticles. As discussed above, $\beta = 4$. The microbead's friction coefficient, f , is calculated from Equation 5 for the known viscosity of the medium, $\eta = 0.001 \text{kg/m/s}$, and the diameter of the Quantum Simply Cellular microbead, $D_c = 8.5 \mu\text{m}$, which leads to $f = 8.01 \times 10^{-8} \text{kg/s}$. Solving for k in Equation 12, a value of $k = \Delta\chi V_m = 2.5 \times 10^{-16} \text{mm}^3$ is obtained. The parameter, k , is intrinsic to the magnetic nanobead and is a constant for a given immunomagnetic label.

Third, the experimental techniques and mathematical models provided here can now be extended to calculate ABC values, within the limits of linearity, through magnetophoretic mobility measurements, of different cell populations. The magnetophoretic mobility versus ABC calibration curve obtained in this study was designed for the quantitation of the CD2 surface molecule expression levels on transformed human fibrosarcoma cells.

In addition to the above work, a reproducibility study was conducted using Quantum Simply Cellular microbeads immunomagnetically labeled with identical and different lots of MACS nanobead antibody reagent. Figure 5 and Table 2 show the histogram and listing of the magnetophoretic mobility results of this study. The CTV data indicate up to a 10% deviation from its averaged magnetophoretic mobility using the same lot of secondary antibody tested on the same and different days. If a different lot of secondary antibody is used, the recorded mobilities deviate up to 20% from the averaged value. Results from a pairwise multiple comparison Tukey test indicate that the differences in these magnetophoretic mobilities are all statistically significant ($P < 0.05$) due to the very low spread and high number of microbead data.

DISCUSSION

A major aim of this study was to demonstrate that a relationship exists between the magnetophoretic mobility of immunomagnetically labeled calibration microbeads and the ABC of those microbeads. This relationship, defined mathematically in Equation 11 and experimentally in Figure 4, indicates that it is theoretically possible to measure the value of the ABC, and presumably cell surface antigen expression levels, of similarly immunomagnetically labeled cell populations.

In addition to developing a relationship between magnetophoretic mobility and ABC, another application of this approach is the experimental measurements of the specific parameters associated with the paramagnetic labels. For example, from the k value obtained from the Quantum Simply Cellular standard curve, it is possible to predict the amount of paramagnetic material (magnetite) in each magnetic nanoparticle ($k = \Delta\chi V_m$, Equations 11 and 12). By knowing the value for the volumetric magnetic susceptibility, $\Delta\chi$, of pure magnetite, one could solve for the volume of magnetite per paramagnetic-antibody bead complex, V_m . The ability to quantitate $\Delta\chi$ and V_m would have a significant impact on the further refinement of immunomagnetic cell separation technology.

CTV analysis may become an important addition to FCM in some applications. Cells in FCM exhibit autofluorescence, which makes it difficult to obtain information on cells with antigen densities lower than three to 5,000 antigens per cell. Immunomagnetic labels can be designed to circumvent this problem. Creating a positive signal (mobility) only requires a strong enough magnetic force operating on the cell or microbead such that it will exhibit some measurable velocity. This is accomplished through using magnetic designs with high magnetic energy gradients combined with paramagnetic binding compounds with sufficiently high k values, thus inducing a velocity detectable by the CTV system.

Another potential advantage of CTV, with respect to FCM, is the stability of the detection system. In FCM, due to shifting of the lasers and detectors and amplification of fluorescent signals, FCM quantitation is only valid if the instrument is recalibrated with these microbead standards each time a cell population is analyzed. Due to the absolute nature of magnetic CTV technology with permanent magnets, there is no need to continuously recalibrate the instrument as long as the antibody-labeling protocol has not been altered. The stability of the CTV magnets has been previously demonstrated (8).

CTV technology was originally designed to measure magnetophoretic mobilities of immunomagnetically labeled cells for performance optimization of continuous magnetic flow sorters. The batch magnetic separation devices, based on cell capture by magnetic substrates, typically are not sensitive to differences in magnetophoretic mobility of the immunomagnetically labeled cells. For continuous flow sorters, the induced mobility is

a fundamental, controlling factor in setting parameters for obtaining a successful separation (6).

Magnetophoretic mobility measurements may have broader applications than magnetic separation and cellular antigen quantitation. The magnetophoretic velocity of single erythrocytes has been investigated by artificially inducing the magnetic state of iron, Fe(III), with sodium nitrite (16–18). Fe(III) can then be reduced to Fe(II) using a medium containing glucose. The reduction of Fe(III) decreases the magnetophoretic mobility of the Fe(III)-rich cells because of the higher magnetic susceptibility of Fe(III) than that of Fe(II). It was proposed by those authors that the diagnosis of patients with glucose-6-phosphate dehydrogenase and reductase deficiency could be made by magnetophoresis.

The long-term applications of using calibration microbeads with CTV technology require further investigations of the correlation between magnetophoretic mobility and ABC. Such a correlation would enable us to accurately predict the conditions for continuous separation processes. In addition, the characteristics of the magnetic particles used as a label (the parameter k) require further investigations to quantitate the influence of the paramagnetic compound on cell motion. It is also desirable to investigate in more detail the individual contributions of each parameter entering Equation 1. These are the goals of the current work in our laboratories.

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