IMMUNOMAGNETIC CELL SEPARATION:
CONTINUED DEVELOPMENT OF FUNDAMENTAL
MODEL OF MAGNETOPHORETIC MOBILITY AND
FURTHER APPLICATIONS

DISSertation

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By

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ABSTRACT

In this study, the theoretical model for magnetophoretic mobility was further developed to include the contribution of intrinsic properties of particles or cells and the influence of the properties of the suspending fluid. Using these improvements, the dependence of the magnetophoretic mobility of unlabeled cells/microspheres and cells/microspheres labeled with colloidal magnetic labels on suspending fluid was used to determine the magnetic susceptibility of cells/microspheres and colloidal labels. The combination of these theoretical improvements, and the experimental studies, makes the methodology developed a universal characterization method.

To explain the dependence of magnetophoretic mobility or fluorescence intensity on concentrations of the affinity labels, a binding model was proposed. Good agreement was found for lymphocytes labeled with anti CD3 antibody conjugates and the model. The data indicated that the conjugation of larger magnetic labels decreases the binding affinity of the antigen antibody interaction and the antibody binding capacity (ABC). A scale up model was then proposed, and experimentally verified on larger scale immunological labeling. This model assists in other projects in our lab focused on the efficient magnetic cell sorting at large scale.
Restricted by high autofluorescence levels, alveolar macrophages, are difficult to analyze using flow cytometry. Immunomagnetically based CTV system was studied to evaluate if CTV can be used as an alternative of flow cytometry on quantitative cellular receptor study. A good agreement between CTV and FCM was found when monocytes were studied. CTV was then successfully used to analyze AM cells for basal receptor expression, and the expression kinetics when LPS was used for activation.

CTV was used to determine if proteins can be immunomagnetically separated via the use of colloidal labels or molecular labels by measuring the magnetism of a labeled protein. Feasibility of the separation process was determined by calculating the retention ratio. It was shown that proteins can be separated immunomagnetically with the use of colloidal labels, such as DM beads, but not with the use of molecular labels used in the study, ferritin.

Immunomagnetic cell separation was also successfully applied to separate *Bacillus cereus* spores from food.
DEDICATED TO MY FAMILY
ACKNOWLEDGMENTS

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>v</td>
</tr>
<tr>
<td>VITA</td>
<td>vii</td>
</tr>
<tr>
<td>List of tables</td>
<td>xiii</td>
</tr>
<tr>
<td>List of figures</td>
<td>xv</td>
</tr>
</tbody>
</table>

## Chapters:

1. Introduction
   1.1 Motivation of current study ................................. 1
   1.2 Scope of the study ........................................... 2

2. Literature Review
   2.1 Introduction on cell separation ............................. 6
   2.2 Introduction on immunological cell separation ............. 7
      2.1.1 Introduction on immunofluorescent cell separation .... 8
      2.1.2 Introduction on immunomagnetic cell separation ....... 9
   2.2 Magnetic labels ............................................... 10
      2.2.1 Magnetic micro labels .................................. 10
      2.2.2 Magnetic colloidal labels .............................. 11
2.2.3  Magnetic molecular labels ........................................12

2.3 Cell’s magnetism ..........................................................13
  2.3.1 Magnetophoretic mobility ......................................14
  2.3.2 Analytical tool for mobility measurement .................15
  2.3.3 Influencing factors on mobility ...............................16
  2.3.4 Implications on controlling magnetophoretic mobility ...17

2.4 Conclusions and future perspective ...............................17

2.5 Acknowledgement ..........................................................18

2.6 Reference ........................................................................19

3.  Establishment and implications of a characterization method for magnetic nanoparticle using Cell Tracking Velocimetry and magnetic susceptibility modified solutions
  3.1 Abstract .......................................................................30
  3.2 Introduction ...................................................................31
  3.3 Theoretical relationships .............................................34
  3.4 Materials and methods ................................................39
  3.5 Results ..........................................................................44
  3.6 Discussions ...................................................................48
  3.7 Acknowledgement ........................................................53
  3.8 Notes .............................................................................53
  3.9 Nomenclature ..............................................................54
  3.10 Reference ....................................................................55

4  The effect of magnetic nano particles on the binding affinity/avidity
  4.1 Abstract .......................................................................77
  4.2 Introduction ...................................................................78
  4.3 Theory ..........................................................................80
4.4 Materials and methods .............................................. 91
4.5 Results ................................................................ 96
4.6 Discussions ........................................................... 101
4.7 Conclusions ........................................................... 104
4.8 Acknowledgement ................................................... 105
4.9 Reference .............................................................. 106

5 Comparison between flow cytometry (FCM) and cell tracking velocimetry (CTV) on quantitative cellular receptor study
5.1 Abstract ................................................................. 127
5.2 Introduction ........................................................... 128
5.3 Materials and methods ............................................. 133
5.4 Results ................................................................ 135
5.5 Discussions & Conclusions ....................................... 139
5.6 Acknowledgement ................................................... 144
5.7 Reference .............................................................. 145

6 Feasibility study of separating proteins using magnetic nano labels
6.1 Abstract ................................................................. 164
6.2 Introduction ........................................................... 165
6.3 Materials and Methods ............................................ 168
6.4 Results ................................................................ 171
6.5 Discussion ............................................................. 172
6.6 Acknowledgement ................................................... 174
6.7 Reference .............................................................. 175
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>List of commercial magnetic cell sorter</td>
<td>24</td>
</tr>
<tr>
<td>2.2</td>
<td>List of commercial magnetic micro labels</td>
<td>25</td>
</tr>
<tr>
<td>2.3</td>
<td>List of commercial magnetic colloidal labels</td>
<td>28</td>
</tr>
<tr>
<td>3.1</td>
<td>Volumetric magnetic susceptibilities of specific elements and compounds</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>(in SI unit system)</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>Size of magnetic nano particles measured by photon correlation spectroscopy</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>(PCS). The mean diameter and standard deviation is based on the result of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>size distribution of several hundred nano particles</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td>Size of magnetic nano particles measured using MiniDAWN®</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>triangle detector. The mean diameter and standard deviation is based on</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the size distribution for several hundred nano particles</td>
<td></td>
</tr>
<tr>
<td>3.4</td>
<td>Size of ProActive Biotin-coated microspheres using a Coulter counter. The</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>mean diameter and standard deviation is based on the results of size</td>
<td></td>
</tr>
<tr>
<td></td>
<td>distribution of several hundred microspheres.</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td>Magnetophoretic mobility value of magnetic microspheres measured under</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>different microsphere concentration in buffer (PBS, 2mM EDTA)</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>CTV results of unlabeled polystyrene microspheres in varying concentration</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>of gadolinium solution</td>
<td></td>
</tr>
<tr>
<td>3.7</td>
<td>CTV results of polystyrene microspheres labeled with 2µL</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>streptavidin-MACS™ (Lot # 5020918049) in varying concentration of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gadolinium solution</td>
<td></td>
</tr>
<tr>
<td>3.8</td>
<td>Numeric values of the intercept, slopes, and quotient of the data presented</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>in Figure 3.5-10. As predicted by equation (11), for the same kind of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>magnetic nanobeads, the intercept and the absolute value of slope becomes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>higher with the higher mobility. The ratio of intercept and the absolute</td>
<td></td>
</tr>
<tr>
<td></td>
<td>value of slope should remain constant for each magnetic particles as</td>
<td></td>
</tr>
<tr>
<td></td>
<td>indicated by equation (11).</td>
<td></td>
</tr>
</tbody>
</table>
For specific lot numbers, the magnetic susceptibility, the mean diameter, and the particle-field interaction parameter, \( \phi \), for the four types of magnetic nanobeads studied. To calculate \( \phi \), the volume of the nanoparticle was assumed to be a perfect sphere using the experimentally measured mean diameter, and suspending fluid has a magnetic susceptibility of water.

Experimentally calculated, values of the particle-magnetic field interaction parameter, \( \phi \), (m³).

Percentage of lymphocytes in peripheral blood cells after culture and Ficoll separation determined by Coulter counter and flow cytometry.

ntiBrite™ beads coated with different PE molecule number.

s of Bacillus cereus spores on MS magnetic column with the procedure recommended by the vendor.

s of Bacillus cereus spores (strain # 6A3) on MS magnetic column with the procedure modified.
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Histograms of the size distribution of Streptavidin MACS™ nanobeads (a), BD™ Imag streptavidin nanobeads (b), EasySep streptaviidn beads (c) and Captivate™ Ferrofluid (d) measured using a 90 Plus Submicron Particle Size Analyzer. A plot of the volumetric susceptibility (A) and viscosity (B) of Optimark® dilutions as a function of Gd$^{3+}$ concentration. The volumetric susceptibility were estimated based on Equation (12), and the viscosity measurements were made on a RFS II rheometer (Rheometrics). A histogram of the experimentally measured magnetophoretic mobility of the magnetic polymeric microspheres at different concentrations. The magnetophoretic mobility was experimentally measured on a Cell Tracking Velocimetry instrument. A histogram of experimentally measured magnetophoretic mobility of ProActive microspheres (5.2 microns) in suspending fluids of different magnetic susceptibility (a), and a scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) in different suspending fluids. The linear line was the regression obtained following equation (5) (b). A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of streptavidin-MACS™ beads (Lot # 5020918049) in different suspending fluids. A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of streptavidin-MACS™ beads (Lot # 5020305031) in different suspending fluids.</td>
<td>64</td>
</tr>
</tbody>
</table>
A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of EasySep™ beads (Lot # 2L226593) in different suspending fluids.

A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of EasySep™ beads (Lot # 3A317176) in different suspending fluids.

A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of BD™ Imag streptavidin nanobeads (Lot # 0000044023) in different suspending fluids.

A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of Captivate™ beads (Lot # 71A1-1) in different suspending fluids.

A comparison of magnetophoretic mobility for T cells labeled with anti CD3 PE and two anti PE magnetic nano particles.

Influence of multiple layers formed after labeling on friction factor on the labeled microspheres.

Potential antibody binding scenarios on cells.

Dot plots of forward light scattering and side light scattering for peripheral blood lymphocytes obtained from Ficoll density gradient (A), after 24 hour culture (B), after 48 hour culture (C), and Ficoll density gradient after 48 hour culture (D).

Size distribution of peripheral blood lymphocytes directly after Ficoll density gradient (in red), after 24 hour culture (in green), and after 48 hour culture (in blue).

Histograms of peripheral blood lymphocytes with mouse anti human CD3 antibody conjugated to FITC (4A), PE (4B), magnetic nano particles in linear scale (4C) and magnetic nano particles in logarithmic scale (4D). In histograms (4A-4C), two peaks are identified as CD3 negative and CD3 positive. In Figure 4D, only one peak is present, which shows that CD3 negative cells have negative mobility.
4.5 Calibration curve generated by using the MicroBCA protein assay, which is linear in the range of 0.5~200µg/mL (5A). Elution curves of anti CD3 DM conjugates and standard protein BSA (Mw=66342) (5B). The elution was conducted on a CL-4B Sepharose™ column and the elutes were analyzed using MicroBCA protein assay; each data point corresponds to a 350 µL elution sample. The peak at the elution volume of 4500~5500 µL corresponds to the elution peak of SA-MACS nanoparticles, which is clearly separated from the peak of BSA, which peaks at around 7900 µL.

Saturation curve and double reciprocal plot for peripheral blood labeled with BD™ anti human CD3-FITC, 6A and 6B, BD™ anti human CD3-PE, 6C and 6D, and BD™ Imag anti human CD3 beads-DM, 6E and 6F.

4.7 FCM histograms, 7A, and calibration curve of QuantiBrite PE Beads, 7B.

Saturation curve and double reciprocal plot for biotinylated polystyrene (5.12 micron) beads labeled with streptavidin-MACS nanobeads, 8A and 8B, and streptavidin-DM nanobeads, 8C and 8D.

4.9 Comparison between predicted $f$ and measured $f$ when immunological labeling is scaled up.

4.10 The effect of cell concentration on the amount of antibodies used when $10^{10}$ cells are labeled.

5.1 Diagram of Cell Tracking Velocimetry (CTV).

5.2 CD14 expression results from flow cytometry for peripheral blood mononuclear cells (PBMCs) before (2A) and after (2B) using MACS™ Monocyte Isolation Kit.

5.3 Side scattering and forward scattering dot plots from flow cytometry of peripheral blood mononuclear cells (PBMCs) before (3A) and after (3B) using MACS™ Monocyte Isolation Kit.

5.4 Size distributions of peripheral blood mononuclear cells (PBMCs) before (green) and after (red) using MACS™ Monocyte Isolation Kit from Coulter counter.
5.5 Fluorescence intensity (FI) of FL1, FL2 and FL3 channels for unlabeled monocytes (A, B, C) and alveolar macrophages (D, E, F). As shown, macrophages have much higher autofluorescence level as compared to monocytes.

5.6 Magnetophoretic mobility of unlabeled alveolar macrophages and monocytes.

5.7 Influence of endocytosis on magnetic labeling of monocytes (A) and alveolar macrophages (B). As shown, when labeling temperature is set to 4°C, neglctable effect of endocytosis was observed.

5.8 Monocytes enriched from peripheral blood mononuclear cells (PBMCs) were labeled with FITC conjugated anti human CD14 antibody and CD14 expression was measured both in CTV (Labeled with anti-FITC MACS™ beads) (A) and flow cytometry (B). Histogram (B) was generated using the gating for certain size range. A clear distinction of CD14 positive cells (monocytes) from CD14 negative cells was observed in both methods and the percentage of positive is close for two approaches.

5.9 Histograms of CD14 expression of enriched monocytes cultured for 2 hours with and without LPS incubation of flow cytometry (A) and CTV (B). In both plots, CD14 expression is upregulated with the incubation together with LPS.

5.10 Histograms of TLR4 expression of enriched monocytes cultured for 4 hours with and without LPS incubation of flow cytometry (A) and CTV (B). In both plots, TLR4 expression is upregulated with the incubation together with LPS. The number of TLR4 on monocytes was calculated to be 7100 (with LPS) and 3700 (without LPS) in CTV, while the number was 6100 (with LPS) and 3100 (without LPS) in flow cytometry.

5.11 Basal expression of mannose, CD14 and TLR4 on alveolar macrophages. As shown in (A), clear distinction from control population was achieved for different receptors. Shown in (B), error bar is the standard deviation of 5 experiments.

5.12 Influence of cultured alveolar macrophage expression of TLR4 (A), mannose (B), and CD14 (C) with and without LPS (100ng/mL).

6.1 Size distribution of coated microspheres measured from Coulter counter
6.2 Protein coated microspheres labeled with streptavidin PE conjugates and the FI measured by flow cytometry (2A), and the relation between FI and the amount of streptavidin PE conjugates (2B).

6.3 Measured FI values for QuantiBrite PE calibration beads at FL2 (3A), and the established calibration curve between FI and the amount of PE molecules (3B).

6.4 Measured magnetophoretic mobility distributions of coated microspheres labeled with streptavidin DM beads (4A) and the relation between mobility and the amount of streptavidin DM beads (4B).

6.5 Measured magnetophoretic mobility distributions of coated microspheres labeled with avidin ferritin.

7.1 The diagram of separation procedure

7.2 Modified separation procedure
CHAPTER 1: INTRODUCTION

1.1 Motivation of current study

Separation of cells via their immunological properties is an important practice in cell biology, medical treatment, and industrial applications. Compared to commonly used flow cytometry (FCM), magnetic cell sorting provides users an easier and potentially cheaper solution. With the continuing development of flow through magnetic sorting system, such as the quadrupole magnetic sorter (QMS) and dipole magnetic sorter (DMS) developed in our group, higher throughput can be obtained with respect to FCM. Some studies have shown the damage of FCM and cell retention type magnetic cell separation on cells, which demands continuing understanding on the separation process.

It has been demonstrated previously that the performance of magnetic cell separation relates directly to how magnetic cells are via the use of different magnetic labels. Magnetophoretic mobility is a parameter used to quantify a cell’s magnetism. An analytical tool named cell tracking velocimetry (CTV) has been established in our group to accurately measure mobilities of cells on a per cell base. Different factors influencing mobility are examined and discussed in previous studies, including antibody binding capacity (ABC), secondary amplification factor, and cell size. With the increasing demand of large scale cell separation for biomedical applications, such as bone marrow transplantation where up to $10^{10}$ cells are processed, a more sophisticated
magnetophoretic mobility model is desired so that prediction of mobilities needed and achievable can be made prior to the labeling step.

1.2 Scope of the study

To tune the mobility for better separation outcome, choice of magnetic labels becomes very crucial. While characterization of magnetic micro labels becomes a standard procedure, characterization of magnetic colloidal or molecular labels still cannot be routinely performed in labs. My work starts with finding an easy and universal way to characterize magnetic colloidal labels to facilitate their easier selection for different applications. Chapter 3 describes the establishment of a method combining experimental approach and theoretical derivation to determine the particle field interaction parameter for four commercial magnetic colloidal labels via the use of CTV and susceptibility modified suspending fluids. With the use of affinity interaction (biotin streptavidin interaction in my study), colloidal labels can be “visualized” on a microsphere, which makes this method applicable to any colloidal or molecular labels. A more complete magnetophoretic mobility model is also introduced and experimentally tested to account for the contribution of intrinsic magnetophoretic mobility, the mobility of unlabeled microspheres or cells.

It has long been found that when the antibody concentration increases, magnetophoretic mobility shows a saturation type phenomenon. Chapter 4 aims to explain this phenomenon, and study the effect of relatively larger magnetic label to the binding of antibody to cellular antigen. Surprisingly with the prevalent use of FCM on binding affinity determination, Scatchard analysis was normally applied to fit the data,
which was based on different working systems. A mathematical model is thus proposed to describe the binding of antibody conjugates to antigens on cell surface. With the use of CTV, size exclusive chromatography, and protein assay, the dependence of mobility to the antibody concentration was established for lymphocytes labeled with CD3 antibody. The result was compared with what were obtained in FCM, where CD3 antibody is conjugated to fluorochromes (FITC or PE). Similar study was also performed on biotinylated microsphere labeled with streptavidin magnetic labels. The influence of conjugation to binding can then be discussed. A scale up model is proposed at the end of the chapter. With the experimentally determined parameters and an independent study of quantifying antibody binding capacity, the predicted outcome of immunological labeling using the scale up model was compared with what was determined experimentally to check the validity of the model.

One of motivations of further development of magnetophoretic mobility model is to solve some real problems faced in biomedical research. Quantification of cellular receptors has become a useful criterion on monitoring the disease status. Alveolar macrophages (AM), being an important player in innate immune response, are known to have strong autofluorescence level, which makes the positively labeled AM cells indistinguishable from the unlabeled AM cells. Effort of biologists and doctors to relate disease status to AM receptor expression level or expression kinetics is hampered by the technical difficulty they face in FCM. With the similarity between immunofluorescence technique and immunomagnetic technique, the potential of CTV being used as “magnetic flow cytometry” is studied in Chapter 5. Comparison between FCM and CTV is first
made with the use of peripheral blood monocytes, which don’t have difficulty when
being analyzed in FCM. Besides comparing CTV with FCM for accuracy in determining
positive percentage, sensitivity of these two techniques is also compared. Antibody
binding capacity is also determined for both techniques and compared. CTV is then
applied on AM cells obtained from bronchoalveolar lavage (BAL) performed on
seemingly healthy donor to study the receptor expression level and kinetics when
lipopolysacharide (LPS) (from E. coli) is used to mimic the invasion of microbes.

Chapter 6 employs the use of CTV to study the feasibility of developing a protein
separation device with the use of nano or molecular labels. Carboxyl modified
microspheres are first coated with biotinylated proteins. The amount of proteins coated
per microsphere is determined via the use of streptavidin-PE and flow cytometry, and the
calibration is done with the use of QuantiBrite® PE beads. Magnetic colloidal label, BD
Imag™ DM beads and paramagnetic protein, ferritin, are then used separately to label
coated microspheres, and the resulted mobility is determined in CTV. The magnetism of
labeled single protein can then be derived, and used to find out the required dimensions
needed for separation, which is compared to dimensions current technology can offer to
determine if this is a feasible approach.

Chapter 7 is an application of immunomagnetic cell separation on Bacillus cereus
spore depletion, which is similar to Bacillus anthracis, with the use of MiniMACS™ and
colloidal magnetic labels. A modified procedure is compared to what is recommended by
the company to try to find an efficient process for food processing.
Chapter 8 summarizes studies done and conclusions made. Future work is proposed at the end of the chapter for better understanding of magnetophoretic mobility, and development of more sophisticated model for easy adjustment of mobility in cell separation.
CHAPTER 2: LITERATURE REVIEW

2.1 Introduction on cell separation

Isolation of cell sub-populations has been a major goal in the research on cell biology [Nadali, 1995], disease diagnostics [Iinuma, 2000], therapeutics [Marini, 1999], and as well as industrial applications [Vogel, 1999; Kempken, 1995]. Different separation methods have been applied based on the classified features of target cells with respect to other cells in a heterogeneous mixture. These separations can be classified as physicochemical, immunologic, and functional separations [Orfao, 1996]. The physicochemical characteristics of cells include cell size, volume, density, light scattering properties, membrane potential, pH, and so on. By far, most of the separations carried out daily are based on cell’s physicochemical properties [Wells, 1989; Pretlow, 1989; Corash, 1986; Pretlow, 1976; Havenith, 1993; Eggleton, 1992]. Just to mention a few, Ficoll-Paque™ density gradient has been a part of standard procedure for enrichment of lymphocytes from peripheral blood [Lehner, 2002]; and centrifugation separation based on difference in cell size is widely used in daily laboratory operations. Functional separation normally is based on whether and how cells withstand different culturing conditions [Jones, 1993]. For instance, we normally culture the enriched peripheral blood lymphocytes after density separation in tissue culture flasks at 37 °C and 5% CO₂ for 48 hours in order to deplete contamination from monocytes, as monocytes are known to be
“sticky”, and are more likely to attach to the surface of flasks. Another example of functional separation is the selection of fused hybridoma cells from myeloma cells based on the ability of cells to grow on HAT (hypoxanthine, aminopterin, and thymidine) containing medium [Olsson, 1980]. Cell separations based on strong bioaffinity interactions have long been used [Polanowski, 2003], but are limited by the number of interactions exist in the nature. Since the development of monoclonal antibody [Kwan, 1980], the use of immunologically based separation is progressively expanding [Dyer, 1992; Wormmeester, 1990].

Because of the specificity and high affinity of antibody-antigen interaction, usually more purified cell population is expected from the immunological separation. At the same time, the cost associated with immunological separation is usually higher compared to other physical separation methods.

2.2 Introduction on immunological cell separation

Immunological cell separation can be performed at the batch or flow through mode [O’Donnell, 2001]. Based on the purpose of the separation, it can be performed as positive selection and negative selection mode. For positive selection, cells we target are the cells of interest; while for the negative selection, cells we target are the cells we don’t want. Positive selection is relatively simpler than the negative selection, as usually only one or two antibodies are needed in the process. On the contrary, negative selection is more complicated, as multiple antibodies are needed to deplete all the other cell types contained. Based on the purpose of cell separation, positive selection and negative selection should be carefully chosen. It has been reported, though, that the membrane
physiology of positively targeted cells is changed following immunofluorescent sorting and retention type, immunomagnetic sorting [Seidl, 1999]. Another study shows the functions of immunomagnetically separated (retention type) CD34 positive stem cells are impaired [Minamiguchi, 2004]. As the result, researchers in biology or biochemistry often prefer negative selection as cells thus obtained are thought as “untouched”. While this may not be true considering the strong hydrodynamic forces flow cytometry has on cells in the stream (group communications, unpublished data), negative selection is still favored in biological community especially for magnetic cell separation as no bead detachment is needed for further studies.

2.2.1 Introduction on immunofluorescent cell separation

Fluorescently-activated cell sorter (FACS, which is widely used) is a per cell based flow through cell sorter, which separates cells based on the fluorescence intensity of cells labeled with antibody fluorochrome conjugates [Pinkel, 1982]. The relative small size of fluorochromes assures the separation be carried out quantitatively [Horan, 1977; Van den Bogert, 1991]. With the combination of excitation lasers and fluorescent dyes, nowadays, a sophisticated flow cytometer can separate cells based on the combination of up to 11 parameters, such as cell size, cell granularity, viability, and cellular antigen expression. Current flow cytometers are capable of sorting cells at the speed as high as several thousand per second. FACS diverts the path of cells by electronic charge based on criteria researchers set. Currently, up to four portions of cells can be obtained following sorting. Immunomagnetic cell separation is a relatively new technique with respect to FACS, and is discussed in detail as following.
2.2.2 Introduction on immunomagnetic cell separation

Compared with FACS, magnetic cell separation is based on the difference in cell’s magnetism between target cells and other cells in the mixture. Normally all the cells we studied are slightly diamagnetic with respect to biological buffer (mainly water) with an exemption of erythrocytes, which can be either diamagnetic or paramagnetic, depending on the oxygenization state of their hemoglobins [Zborowski, 2003] and magnetotactic bacteria, which contain small magnetic particles within the cell [Blakemore, 1975]. So the magnetic cell separation based on cell’s intrinsic magnetism is only limited to erythrocytes and magnetotactic bacteria, and will not be discussed in detail in this paper. To differentiate cells in their magnetism, target cells are labeled specifically with magnetic labels via antibody-antigen interaction or other bioaffinity interactions, such as biotin-streptavidin interaction.

Current commercialized magnetic cell separation systems are mostly operated at the batch mode, which are listed in Table 1. These separation systems are mostly based on whether or not cells can be retained in the column or to the vessel wall where magnetic field applies.

Our lab has developed two flow through magnetic sorters, dipole magnetic sorter (DMS) and quadrupole magnetic sorter (QMS) [Moore, 1998; Chalmers, 1998A]. They separate cells based on the distances they travel under the magnetic energy gradient, which are directly related to the cell’s magnetism after labeling. These systems can provide higher or comparable throughput compared to FACS.
2.3 Magnetic labels

Based on the difference in size, magnetic labels can be classified as micro, colloidal, and molecular labels. In most cases, the magnetic properties of the labels are caused by the presence of small particles of magnetite (Fe₃O₄) or maghemite (γ-Fe₂O₃). These magnetic labels also can be classified as paramagnetic and superparamagnetic labels.

Superparamagnetic labels, whose magnetic components are too small to form a single domain, are attracted by a magnetic field but retain no residual magnetism once the field is removed. It has been shown that at room temperature, superparamagnetic labels have higher magnetic susceptibility than the paramagnetic labels. Another thing that makes superparamagnetic labels more attractive is that magnetically labeled cells can be easily suspended outside of the field, as the labels don’t have remnant magnetism. There are some criteria for magnetic labels, especially synthesized labels, to be used in bioseparations, i.e., they should be chemically stable, and the iron content of the labels won’t leak out to influence cells. So these synthesized magnetic labels are usually composed of iron oxide core with a biocompatible shell. Also, in most applications, nonspecific binding or endocytosis is discouraged, surface properties and size of these labels should be strictly controlled.

2.3.1 Magnetic micro labels

Magnetic particles of 1 ~ 5 microns in diameter, such as Dynabeads (available in 2.8, 4.5, and 5.0 microns, activated and non-activated forms, Dynal AS, Oslo, Norway)
are considered micro labels, and they are on the order of a cell diameter. There are a number of magnetic micro labels available commercially, some of which are listed in Table 2. Dynabeads are prepared from monosized polystyrene particles, which are magnetized by an in situ formation of ferromagnetic material inside the pores (“activated swelling” technique) [Prestvik, 1997]. Because of the relatively large size, these micro labels exert higher forces in the field, which can separate the labeled cells faster, and even in a weak magnetic field. Most of these micro labels are claimed by their manufacturer to be superparamagnetic, which makes sense because in most labels magnetic component is spread through the sphere.

2.3.2 Magnetic colloidal labels

Typically, colloidal magnetic labels are 50 ~ 400 nm in diameter, some of the commercially available colloidal labels are listed in Table 3. These colloidal labels are generally considered as superparamagnetic. The colloidal behavior of the labels makes them faster to react with cells, and usually no mixing is needed for the binding process. It has been demonstrated that the amount of colloidal labels bound onto cells is directly related to the antigen expression level of cells, which makes immunomagnetic separation a quantitative sorting, similar to FACS [Chalmers, 1998B]. These colloidal labels can be synthesized using a variety of methods, including coprecipitation of ferric and ferrous salt in the presence of sodium hydroxide, and coating polymer, such as dextran (used for synthesis of 50 nm MACSTM beads, Miltenyi Biotec, Germany) [Molday, 1982; Miltenyi, 1990], coating the preformed iron oxide core (by coprecipitation method) with
polysaccharides or synthetic polymers [Gruttner, 1997], microwave irradiation heating of an aqueous solution containing ferric salt, and polymer [Liao, 2001], and etc.

Besides the widely used synthesized magnetic nano particles, recently, magnetic liposomes, magnetic quantum dots and magnetic dendrimers appear to draw our attention [Langereis, 2004; Mackowski, 2003; Jain, 2003], which makes magnetic cell separation applicable due to the increased ease of managing surface and reducing the toxicity to cells.

2.3.3 Magnetic molecular labels

Erbium (Er$^{3+}$) is a well known paramagnetic element, and has been used to label cells in the past via mainly ionic interaction [Zborowski, 1992]. However, its use is limited because erbium binds to almost everything on the cell, and thus its specificity is relatively low. Gadolinium also is a paramagnetic element, which does not undergo protein binding in vitro. Gadolinium salts have been used as a contracting agent in magnetic resonance imaging (MRI).

Naturally occurring ferritin stores iron in mammals [Al-Massad, 1992]. It consists of a hollow protein shell of approximately 13 nm in diameter, with a cavity of approximately 7 nm, where iron is deposited [Zborowski, 2003].

Magnetic molecular labels can be combined with colloidal labels to form a magnetic colloidal label, such as the gadolinium loaded immunoliposomes.
The debate on choice between magnetic micro labels and magnetic colloidal labels has been around for some time. There is no clear criteria on which is better. Obviously, to be recognized as “magnetically positive”, fewer labels are needed for micro labels than colloidal or molecular labels, as micro labels offer more force when in a magnetic energy gradient. For cells with low antigen expression, micro labels should be chosen. At the same time, in order to use the separated cells for other studies, micro labels should be detached via extra treatment, which makes the process more complicated and lengthy. Generally, micro labels work better for depletion (negative selection), because lower frequency of false negative is expected. On the contrary, colloidal labels work better for enrichment (positive selection), because the frequency of false positive is lower.

2.4 Cell’s magnetism

In order to quantify how magnetic cells are after immunomagnetic labeling, a parameter needs to be defined. This parameter should provide a realistic way of measurement, and an easy way of comparison between different magnetic systems. As can be figured out, it is a factor relating to cell’s characteristic properties, and magnetic labels’ characteristic properties. Magnetophoresis is the motion of an electrically neutral body in a viscous medium when exposed to a magnetic energy gradient [McCloskey, 2000]. The speed of cells moving in the field provides a measurement of cell’s magnetism, which is called magnetophoretic mobility as will be discussed below.
2.4.1 Magnetophoretic mobility

Analogous to electrophoretic mobility, magnetophoretic mobility can be described as the ratio of driving force to the friction, and can be described as in equation (1).

\[ m = \frac{D}{f} \quad (1) \]

where \( m \), \( D \), and \( f \) represent the magnetophoretic mobility, driving force, and friction. It can be shown that the driving force is the product of relative volumetric magnetic susceptibility with respect to suspending solution, \( \Delta \chi \), and the volume of the particle, \( V \). For slow moving particles, friction term can be described using Stoke’s equation [Reddy, 1996].

\[ f = 3\pi d \eta \quad (2) \]

where \( d \), \( \eta \) are the diameter of the particle, and viscosity of the suspending fluid respectively.

For unlabeled cells, the intrinsic magnetophoretic mobility is derived as equation (3).

\[ m = \frac{\Delta \chi \cdot V}{3\pi d \eta} = \frac{\phi}{3\pi d \eta} \quad (3) \]
\( \phi \) is called the particle field interaction parameter, which expresses characteristics of certain particle. When cells are labeled with magnetic labels, equation (3) should be modified to

\[
m = \frac{\phi + N \cdot \phi_{\text{label}}}{3\pi d' \eta}
\]

(4)

where \( N, \phi_{\text{label}}, \) and \( d' \) are the number, particle field interaction parameter of magnetic labels, and the diameter of then formed cell label complex.

When magnetic colloidal labels are used, equation (4) can be modified to relate the mobility to antigen expression level [McCloskey, 2001].

\[
m = \frac{\phi + ABC \cdot \psi \cdot n_3 \cdot \phi_{\text{label}}}{3\pi d' \eta}
\]

(5)

where \( ABC \) is called the antibody binding capacity, and it represents the antigen expression level directly. \( \psi \) is the secondary amplification factor for multiple step labeling, and equals one if only one step labeling is involved. \( n_3 \) is the number of magnetic particles conjugated per antibody.

2.4.2 Analytical tool for mobility measurement

Gill et al., 1960 determined the velocity of particles in a magnetic field via the use of stopwatch and microscope. Other researchers have used video technique to track the cells, and the cell trajectory was determined based on some model fitting [Bahaj, 1989; Takayusu, 1982]. In our lab, we have developed an analytical tool, called Cell Tracking
Velocimetry (CTV) to measure the mobility of cells with the use of a well defined magnetic energy gradient [Chalmers, 1999]. In brief, the movement of cells or particles in the field is captured by a video camera, and the digitalized images are analyzed with the algorithm adapted from Particle Tracking Velocimetry (PTV) [Guezennec, 1994]. The algorithm determines the location of a particle in each frame by calculating the most probable path of the particle from five consecutive images. The velocity of the particle in the middle frame is calculated as the average velocity in five frames. It has been proven that CTV can be used to accurately measure magnetism of cells and paramagnetic particles [Moore, 2000]. Very little day to day variation and user to user variation is found [Nakamura, 2001].

2.4.3 Influencing factors on mobility

McCloskey et al. 2003A discussed influencing factors on magnetophoretic mobility, including antigen binding capacity, secondary amplification factor (for multistep labeling), particle field interaction parameter of magnetic labels, and cell size, as shown in equation (5). Moore et al., 2004 further shows the dependence of mobility on the suspending fluid, and this dependence can be mathematically expressed as equation (6) for unlabeled particles

\[
m \cdot \eta = \frac{\chi \cdot V}{3 \pi d} - \frac{V}{3 \pi d} \cdot \chi_f \tag{6}
\]

where \( \chi_f \) is the volumetric magnetic susceptibility of the suspending fluid.
It has also been found that magnetophoretic mobility relates to the amount of antibodies used [Comella, 2001; Chosy, 2003]. Taking consideration of classical receptor ligand interaction theory, the amount of antibody used together with the total antigen expression level influence the amount of antibodies bound, or the antibody binding capacity, whose influence on mobility is studied previously.

2.4.4 Implications on controlling magnetophoretic mobility

Magnetophoretic mobility has been shown to directly influence the outcome of magnetic cell sorting [McCloskey, 2003B]. In retention type batch magnetic separation devices, the mobility of positively labeled cells should be larger than a certain threshold in order to be considered “positive”. Also, Comella et al., 2001 and Melnik et al., 2001 show that in MiniMACS™ system, positively labeled cells can be permanently retained in the column if cells have too high magnetism. In flow through cell separation systems, mobility can be tuned to achieve high throughput and high separation efficiency [Williams, 1999].

2.5 Conclusions and future perspectives

It has been shown in the past that magnetophoretic mobility should be treated as a control factor to optimize the separation process. Among factors influencing magnetophoretic mobility, choice of magnetic labels is the first factor users should consider for best results. With labels of a variety of different size and composition available either commercially or through research collaborations, no well established method can be found on their characterization. In the future, it is predicted that with the
mathematical model of mobility becoming more complete, a good understanding of controlling factors will lead to more efficient immunomagnetic cell sorting, which finds magnetic cell separation more applications in bioprocessing, and biomedical treatments, such as bone marrow transplantation.

2.6 Acknowledgements

This work has been supported by the National Science Foundation (BES-9731059 to J.J.C. and CTS-0125657 to P. S. W.) and the National Cancer Institute (R01 CA62349 to M.Z. and R33 CA81662-01 to J.J.C.).
2.7 Reference


Zborowski, M., et al., *Quantitative separation of bacteria in saline solution using lanthanide erbium (III) and a magnetic field*, Journal of General Microbiology, 1992, **138**(1), 63-8.


<table>
<thead>
<tr>
<th>Name</th>
<th>Separation mode</th>
<th>Description</th>
<th>Manufacturer/supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>MiniMACSTM, MidiMACSTM, QuadroMACSTM, VarioMACSTM, SuperMACSTM</td>
<td>Flow through</td>
<td>Ferrous spheres clustered column hold positive cells, direct contact of ferrous spheres with cells</td>
<td>Miltenyi Biotec, Germany</td>
</tr>
<tr>
<td>MPC-1, MPC-2, MPC-6, MPC-E-1, MPC-E-6, MPC-M</td>
<td>Batch</td>
<td>No direct contact of labeled cells with magnets</td>
<td>Dynal, Oslo, Norway</td>
</tr>
<tr>
<td>OptiCell® (with the use of Dyan®beads)</td>
<td>Batch (for culture plate size), small processing volume</td>
<td>No direct contact of labeled cells with magnets</td>
<td>OptiCell, OH, USA</td>
</tr>
<tr>
<td>Imag™</td>
<td>Batch</td>
<td>No direct contact of labeled cells with magnets</td>
<td>BD Biosciences, Pharmingen, CA, USA</td>
</tr>
<tr>
<td>MAGSORT</td>
<td>Flow through</td>
<td>Dividing into up to 15 individual fractions</td>
<td>SHOT, Greenville, IN, USA</td>
</tr>
<tr>
<td>Cell enrichment column &amp; MagCellect</td>
<td>Batch/flow through</td>
<td></td>
<td>R&amp;D systems, USA</td>
</tr>
<tr>
<td>EasySep (without column) &amp; StemSep (with column)</td>
<td>Batch/flow through</td>
<td></td>
<td>Stemcell technologies, Canada</td>
</tr>
<tr>
<td>Magnetic Separator (2, 10 microcentrifuge tubes, 96 well plate)</td>
<td>Batch</td>
<td>No direct contact of labeled cells with magnets</td>
<td>Cortex Biochem, Inc., USA</td>
</tr>
<tr>
<td>MagNest®</td>
<td>Batch</td>
<td>No direct contact of labeled cells with magnets</td>
<td>Immunicon, Huntingdon Valley, PA, USA</td>
</tr>
<tr>
<td>LifeSep™ and MixSep™</td>
<td>Batch</td>
<td>No direct contact of labeled cells with magnets</td>
<td>Dexter Magnetic Technologies, Inc., USA</td>
</tr>
<tr>
<td>Magnetic Rack</td>
<td>Batch</td>
<td>No direct contact of labeled cells with magnets</td>
<td>New England BioLabs® Inc., USA</td>
</tr>
</tbody>
</table>

Table 2.1 List of commercial magnetic cell sorters
<table>
<thead>
<tr>
<th>Name</th>
<th>Diameter (µm)</th>
<th>Composition</th>
<th>Immobilized ligands</th>
<th>Surface modification</th>
<th>Manufacturer/supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>COMPEL™</td>
<td>3, 6, 8 (c.v. ≤ 5%)</td>
<td>Magnetite dispersed in a polymer matrix, and encapsulated by polymer coating</td>
<td>Any protein</td>
<td>COOH-modified surface</td>
<td>Bangs Laboratories, Inc.</td>
</tr>
<tr>
<td>Classical magnetic microsphere</td>
<td>~1</td>
<td>Magnetite (12–60%) dispersed throughout the beads</td>
<td>-COOH or –NH₂ modified</td>
<td></td>
<td>Bangs Laboratories, Inc., USA</td>
</tr>
<tr>
<td>Encapsulated magnetic microsphere</td>
<td>1.63</td>
<td>Classical magnetic microspheres with added outer shell of pure polymer</td>
<td>-COOH or –NH₂ modified</td>
<td></td>
<td>Bangs Laboratories, Inc., USA</td>
</tr>
<tr>
<td>CELLection™ Dynabeads®</td>
<td>4.5</td>
<td>Polymer shell (polystyrene) with magnetite core</td>
<td>Recombinant streptavidin (rSA)</td>
<td>Via DNA linker (for easy detachment)</td>
<td>Dynal® Biotech, Norway</td>
</tr>
<tr>
<td>Dynabeads® M-450 Epoxy/Tosyl activated</td>
<td>4.5</td>
<td>Polymer shell (polystyrene) with magnetite core</td>
<td>Any proteins</td>
<td>Hydrophobic naked beads with glycidyl ether (epoxy) reactive groups or –SH modified</td>
<td>Dynal® Biotech, Norway</td>
</tr>
<tr>
<td>Dynabeads®</td>
<td>4.5</td>
<td>Polymer shell (polystyrene) with magnetite core</td>
<td>Antibodies, and secondary antibodies</td>
<td></td>
<td>Dynal® Biotech, Norway</td>
</tr>
<tr>
<td>Sera-Mag®</td>
<td>1</td>
<td>Polymer shell with iron core</td>
<td>Any proteins, streptavidin, Oligo(dT)</td>
<td>Carboxylate-modified</td>
<td>Seradyn, IN, USA</td>
</tr>
</tbody>
</table>

Continued at page 25
<table>
<thead>
<tr>
<th>Product</th>
<th>Description</th>
<th>Additional Components</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>XM-200 XMO-207 XMS-203</td>
<td>3.5</td>
<td>Oligo(dT), streptavidin</td>
<td>Abgene, U.K.</td>
</tr>
<tr>
<td>MagaBeads®</td>
<td>Ultra-pure paramagnetic iron oxide (Fe3O4) suspended and uniformly entrapped in polymer supports, such as polysaccharides, acrylic polymers, and copolymers</td>
<td>Secondary antibodies, enzymes, and bioactive compounds</td>
<td>Cortex Biochem, Inc., USA</td>
</tr>
<tr>
<td>MPG®</td>
<td>5</td>
<td>Porous (50nm) borosilicate glass with iron oxide</td>
<td>CPG Inc., USA</td>
</tr>
<tr>
<td>BioMag®</td>
<td>1.8</td>
<td>Secondary antibodies, anti fluorescein, protein A/G, streptavidin, oligo(dT)</td>
<td>Polysciences, Inc., Warrington, PA, USA</td>
</tr>
<tr>
<td>SPHERO™</td>
<td>2.0–24</td>
<td>A layer of magnetite and polystyrene coated onto polystyrene core</td>
<td>Spherotech, Inc., USA</td>
</tr>
<tr>
<td>Magnetic cellulose/agarose particles</td>
<td>3</td>
<td>Oligo(dT), biotin, streptavidin, DEAE, CM, phospho</td>
<td>Vector Laboratories, Inc., USA</td>
</tr>
</tbody>
</table>

Continued at page 26
Continued from page 25

<table>
<thead>
<tr>
<th>AGOWA® mag particles</th>
<th>&lt; 53, 80% 5~10</th>
<th>Fe₃O₄ core coated with polymer</th>
<th>DNA binding</th>
<th>Irregular</th>
<th>Agowa, Berlin, Germany</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic beads</td>
<td>1</td>
<td></td>
<td>Streptavidin, secondary antibodies, protein A/G</td>
<td></td>
<td>New England BioLabs® Inc., USA</td>
</tr>
<tr>
<td>Coated polystyrene magnetic particles</td>
<td>1~10</td>
<td>A layer of iron oxide and polystyrene coated onto the core of particles</td>
<td>Avidin, streptavidin, secondary antibody, protein A/G</td>
<td></td>
<td>G.Kisker, USA</td>
</tr>
</tbody>
</table>

Table 2.2 List of commercial magnetic micro labels
<table>
<thead>
<tr>
<th>Name</th>
<th>Diameter (nm)</th>
<th>Composition</th>
<th>Immobilized ligands</th>
<th>Manufacturer/supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferrofluids</td>
<td>70, 135, 175</td>
<td>Goats anti-mouse (rabbit) IgG, streptavidin</td>
<td></td>
<td>Immunicon, Huntingdon Valley, PA, USA</td>
</tr>
<tr>
<td>MACSTM microbeads</td>
<td>50</td>
<td>Dextran coats iron oxide core</td>
<td></td>
<td>Miltenyi Biotec, Bergisch Gladbach, Germany</td>
</tr>
<tr>
<td>Imag™-DM, MSC</td>
<td>100–450</td>
<td>Streptavidin, CD3, CD4, CD8, CD14, CD19, CD25, CD45, CD56, anti APC, anti PE, mouse IgG</td>
<td>BD Biosciences, Pharmingen, CA, USA</td>
<td></td>
</tr>
<tr>
<td>SupraMag™</td>
<td>200–1000</td>
<td>Any proteins, Amino, Chloromethyl, Sulphhydrly surface modified</td>
<td></td>
<td>PolyMicrospheres, USA</td>
</tr>
<tr>
<td>MagCellect ferrofluid</td>
<td>150</td>
<td>Secondary antibodies, streptavidin</td>
<td></td>
<td>R&amp;D systems, USA</td>
</tr>
<tr>
<td>SPHERO™</td>
<td>100–900</td>
<td>Any proteins, Carboxyl modified</td>
<td></td>
<td>Spherotech, Inc., USA</td>
</tr>
<tr>
<td>EasySep™</td>
<td></td>
<td>streptavidin</td>
<td></td>
<td>Stemcell technologies, Canada</td>
</tr>
<tr>
<td>Magnetizable nanoparticles</td>
<td>250</td>
<td>Dextran based/silicon based</td>
<td>Streptavidin, carboxyl modified</td>
<td>G.Kisker, USA</td>
</tr>
</tbody>
</table>

Table 2.3 List of commercial magnetic colloidal labels
CHAPTER 3: ESTABLISHMENT AND IMPLICATIONS OF A CHARACTERIZATION METHOD FOR MAGNETIC NANOPARTICLE USING CELL TRACKING VELOCIMETRY AND MAGNETIC SUSCEPTIBILITY MODIFIED SOLUTIONS

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Contents A methodology for determining the field interaction parameter of magnetic nanoparticles is presented along with actual experimental measurements of four different types of magnetic nanoparticles. The implication of differences in magnitude of field interaction parameters is discussed and experimentally demonstrated by comparing labeled human lymphocytes.
3.1 Abstract

Magnetic micro and nano particles conjugated to affinity labels have become a significant, commercial reagent. It has been demonstrated that the performance of cell separation systems using magnetic labels is a function of the magnitude of the magnetic force that can be generated through labeling. This magnetic force is proportional to the number of magnetic particles bound to the cell, the magnetic energy gradient, and the particle-field interaction parameter. This particle-field interaction parameter, which is the product of the relative volumetric, magnetic susceptibility and the volume of the micro or nano particle, is a fundamental parameter which can be used to characterize the magnetic particles. An experimental technique is presented which measures the volumetric magnetic susceptibility of particles through the use of susceptibility modified solutions and an experimental instrument, Cell Tracking Velocimetry, CTV. Experimental studies were conducted on polystyrene microspheres alone and bound to four different magnetic nanoparticles. The experimentally determined values of the magnetic susceptibility of the polystyrene microspheres are consistent with values found from literatures. Consequently, magnetic susceptibility measurements of these polystyrene microspheres bound with the magnetic nanoparticles combined with particle size measurements using commercial dynamic light scattering instrument allowed estimates of the particle-field interaction parameter to be made for four commercial, magnetic nano particles. The value found for MACSTM beads is close to what is reported from an independent study. The values for MACSTM beads and ImagTM beads are found to agree with what is observed
from experiments. Finally, an experimental demonstration of the impact that differences in this field interaction parameter has on the labeling of human lymphocytes is presented.

3.2 Introduction

Separation of specific cells of interest using immunomagnetic labels has become a significant preparative technology in clinical and research settings. A recent computer search of the biological literature (Medline) indicated over 400 articles in the last 5 years, referencing magnetic cell separations as at least a part of the experimental protocols. A few examples, which demonstrate the range in the reported types of cells separated, include: the separation of CD34+ peripheral blood stem cells (PBSC) [Despres, 2000], the separation of rare, circulating cancer cells from patients [Racila, 1998], the separation of fetal cells from peripheral maternal blood [Geifman-Holtzman, 2000], the separation of glial cells from central nervous tissue [Wright, 1997], and the separation of microorganisms from foods [Uyttendaele, 2000].

A majority of these reported separations have been conducted using commercial, cell retention devices such as the MiniMACS™ system (Miltenyi Biotec, Bergisch Gladbach, Germany). One can evaluate the performance of these immunomagnetic cell separation systems by making the following divisions: 1) the ability of the magnetic separation device to retain all the paramagnetically labeled cells, 2) the specificity of the paramagnetic labels for the target cells, and 3) the ability of the paramagnetic labels to induce a magnetic force on the cells sufficient to retain the labeled cells within the cell retention device.
A large range in type of magnetic labels is available either commercially or through research collaborations. While they usually consist of some sort of affinity tag (typically an antibody or streptavidin) conjugated to the magnetic particles, the size of these particles can range from the size of a cell, such as 5 microns (i.e. Dyna M-500 beads, Dynal AS, Oslo, Norway), to a size not much bigger than an antibody, on the order of 50 nanometers, (MACS™ beads, Miltenyi Biotec, Bergisch Gladbach, Germany). Since the magnetic force generated when a magnetic particle is in a magnetic energy gradient is directly proportional to the product of the volume of magnetic material and the material’s magnetic susceptibility relative to suspending fluid, it follows that magnetic particles of 5 microns in diameter create orders of magnitude more force than magnetic particles of 50 nanometers, assuming they have similar material composition. What is not necessarily obvious is that the degree to which a cell is saturated with magnetic particles also significantly affects the force on a labeled cell, and subsequently, the performance of the cell separation system.

With the use of magnetic nanoparticles, it has been demonstrated that it is possible to relate a labeled cell’s magnetism (expressed as magnetophoretic mobility, the cells’ magnetically induced velocity divided by the magnetic field energy gradient) to the expression level of cell surface markers [McCloskey, 2000]. Since most cells are intrinsically diamagnetic [Zborowski, 2003], magnetophoretic mobility measurement of immunomagnetically labeled cells also presents the possibility of characterizing the expression level of surface markers on cells with strong autofluorescence and/or low surface marker expression levels [Tchikov, 1999]. However, to routinely make such
highly sophisticated measurements on a wide variety of cell types and cell states, it requires an improved characterization method for magnetic nano particles.

Commercially available magnetic nano particles typically consist of a polymer, such as dextran or starch, and magnetite and/or other iron oxides [Safarik, 1999]. Table 1 presents the volumetric, magnetic susceptibility (in S.I. units system) of a number of common compounds. Several important points should be noted: first, in addition to water, most organic compounds have a negative magnetic susceptibility (i.e. they are diamagnetic). Second, the volumetric, magnetic susceptibility of various magnetic materials can vary over many orders of magnitude. Third, the oxidation state of iron can have a significant effect on the iron oxide’s magnetic susceptibility. These factors, among others (including the very small size), make accurate, theoretical estimates of the magnetic susceptibility of magnetic nano particles very difficult.

A number of experimental techniques to measure the magnetic susceptibility of particles on the order of microns, or larger, have been reported. These methods include the vibrating sample magnetometer, which provides bulk average values [Moore, 2000]; the calculation of magnetic susceptibility from velocity measurements of the particles in a well defined magnetic energy gradient [Reddy, 1996]; and calculation of the magnetic susceptibility of cells by varying the magnetic susceptibility of the suspending fluid [Russell, 1987].

In this manuscript a procedure is presented which determines the magnetic susceptibility of magnetic particles, on the size scale of 50 -200 nanometers, using a combination of experimental measurements and mathematical derivations. Independent measurements of size were also made which allows the “particle-field interaction
parameter” to be determined. This particle-field interaction parameter, which is the product of the relative volumetric, magnetic susceptibility of the particle with respect to the suspending fluid and the actual volume of the magnetic particle, is a fundamental parameter which has been defined as the proportionality factor between the force acting on the particle and the magnetic field strength [Zborowski, 2002].

3.3 Theoretical Relationships

3.3.1 Magnetophoretic Mobility

When a magnetically susceptible material is placed in a magnetic energy gradient, a magnetic force, \( F_{\text{mag}} \), is induced, which is defined by the following relationship:

\[
F_{\text{mag}} = \Delta \chi V_m \nabla \left( \frac{B^2}{2\mu_0} \right)
\]  

(1)

where \( \Delta \chi \) is the difference in volumetric magnetic susceptibility between the material of interest, \( \chi_m \), and the suspending fluid, \( \chi_f \), \( V_m \) is the volume of the material of interest, \( B \) is the magnetic field induction, and \( \mu_0 \) is the magnetic permeability of free space.

Several significant assumptions were made in Equation (1). First, it is assumed that \( \chi_m \) is a constant (the field strength is sufficiently low that the magnetic material is below its saturation value; See Zborowski et al., 2002, for more details). Second, in the derivation that follows, \( V_m \) will refer to the total volume of the material, and accordingly, \( \chi_m \) refers to an averaged susceptibility of the material.

For a microsphere (or cell) moving in a viscous fluid, the movement will be opposed by a hydrodynamic drag force. Since typically, the movement of the
microsphere as a result of a magnetic force is sufficiently slow [Reddy, 1996], Stoke’s law applies and the drag force for a sphere is given by:

$$F_d = 3\pi \eta d^3$$  \hspace{1cm} (2)

where \(d\) is the diameter of the microsphere, \(\eta\) is the viscosity of the suspending fluid, and \(\nu\) in this case is the induced velocity of the microsphere in the field.

By balancing \(F_{mag}\) and \(F_d\), the induced velocity of the microsphere in the field, \(\nu\) is given by:

$$\nu = \frac{(\chi_{sphere} - \chi_f) \cdot V_{sphere}}{3\pi d_{sphere} \eta} \left(\nabla \cdot \frac{B^2}{2\mu_0}\right)$$ \hspace{1cm} (3)

This induced velocity, \(\nu\), can be divided by \(S_m\), the magnetic energy gradient, equal to \(\nabla \left(\frac{B^2}{2\mu_0}\right)\), to obtain the magnetophoretic mobility, \(m\), which only relates to the properties of microspheres and the suspending fluid:

$$m = \frac{(\chi_{sphere} - \chi_f) \cdot V_{sphere}}{3\pi d_{sphere} \eta} \left(3\pi d_{sphere} \eta\right)$$ \hspace{1cm} (4)

2.3.2 Determination of the volumetric magnetic susceptibility of the microspheres, \(\chi_{sphere}\).

When the same microspheres are suspended in fluids of different magnetic susceptibilities, \(\chi_f\), and subjected to a magnetic energy gradient, \(S_m\), Equation (4) predicts different magnetophoretic mobilities. Rearranging Equation (4), one obtains:

$$m \cdot \eta = \frac{\chi_{sphere} \cdot V_{sphere}}{3\pi d_{sphere}} - \frac{V_{sphere}}{3\pi d_{sphere}} \cdot \chi_f$$ \hspace{1cm} (5)
If one were to conduct a set of experiments where the magnetophoretic mobility, \( m \), was measured when the same microspheres were suspended in fluids of different, known magnetic susceptibility, \( \chi_f \), and then plotted in the form of \( m \cdot \eta \) as a function of \( \chi_f \), Equation (5) predicts that a linear relationship would be obtained. It follows that the quotient of the \( y \)-intercept and slope is the magnetic susceptibility of the sphere, \( \chi_{sphere} \).

3.3.3 Determination of the volumetric magnetic susceptibility for nanoparticles, \( \chi_{nano} \).

When magnetic nanoparticles are used, the magnetic force on microspheres (or cells) labeled with nanoparticles can be represented by a modified form of Equation (1):

\[
\overrightarrow{F}_{mag} = \left[ N_{nano} \cdot \left( \chi_{nano} - \chi_f \right) \cdot V_{nano} + (\chi_{sphere} - \chi_f) \cdot V_{sphere} \right] \cdot \nabla \left( \frac{B^2}{2\mu_0} \right) \tag{6}
\]

where \( N_{nano} \) is the number of magnetic nano particles bound per microsphere, \( \chi_{nano} \) is the volumetric magnetic susceptibility of the magnetic nano particles, and \( V_{nano} \) is the volume of the nano particles.

Similarly, the induced velocity of labeled microspheres in the magnetic field is given by Equation (7).

\[
\nu = \frac{N_{nano} \cdot \left( \chi_{nano} - \chi_f \right) \cdot V_{nano}}{3\pi d_{sphere} \eta} \cdot \left| \nabla \left( \frac{B^2}{2\mu_0} \right) \right| + \frac{(\chi_{sphere} - \chi_f) \cdot V_{sphere}}{3\pi d_{sphere} \eta} \cdot \left| \nabla \left( \frac{B^2}{2\mu_0} \right) \right| \tag{7}
\]

Here the term, \( (\chi_{nano} - \chi_f) \cdot V_{nano} \) is the particle-field interaction parameter, \( \phi \), defined previously and it is assumed that the binding of the nano particles onto the microsphere has no effect on the microspheres diameter.

Again, equation (7) can be converted to (8).
\[
m = \frac{N_{nano} \cdot (\chi_{nano} - \chi_f) \cdot V_{nano}}{3\pi d_{\text{sphere}} \eta} + m_0
\]  

(8)

where \( m_0 = \frac{(\chi_{\text{sphere}} - \chi_f) \cdot V_{\text{sphere}}}{3\pi \eta d_{\text{sphere}}} \), which is the magnetophoretic mobility of the unlabeled microspheres.

If one were to conduct experiments with the identically labeled microspheres in suspending fluids of different magnetic susceptibility, one can rearrange Equation (8) to obtain a linear relationship with the experimentally measured mobility as a function of the magnetic susceptibility of the fluid:

\[
(m - m_0) \cdot \eta = \frac{\chi_{nano} \cdot N_{nano} \cdot V_{nano}}{3\pi d_{\text{sphere}}} - \frac{N_{nano} \cdot V_{nano}}{3\pi d_{\text{sphere}}} \cdot \chi_f
\]  

(9)

When plotted in the form of \((m - m_0) \cdot \eta\) as a function of \(\chi_f\), Equation (9), predicts that a linear relationship would be obtained. It follows that the quotient of the y-intercept and slope is the magnetic susceptibility of the nanosphere, \(\chi_{nano}\).

As stated after the introduction of Equation (1), the previous derivations have been based on the assumption that the magnetic field strength is sufficiently low that the magnetically susceptible of the material (microsphere, nanoparticle, or suspending fluid) is sufficiently below its saturation value. In the specific study presented below, the experimental apparatus used, Cell Tracking Velocimetry, CTV, has a magnetic field strength in the region of analysis ranging from 1.38 to 1.15 T. Such a high magnetic field strength is above the saturation level of iron compounds (but not the other materials) used in the commercial nanoparticles being analyzed. Consequently, the following changes in the relationships are needed. Specifically, Equation (1) now becomes:
\[
\vec{F}_{\text{mag}} = \left( \frac{\mu_0 M_{s,nano}}{B} - \chi_f \right)V_m \sqrt{\frac{B^2}{2\mu_0}}
\]  
(10)

where \( M_{s,nano} \) is the volume-averaged saturation magnetization for the whole nano particle which is a constant. Equations (6) and (9) are also changed and become:

\[
\vec{F}_{\text{mag}} = \left[ \left( \frac{\mu_0 M_{s,nano}}{B} - \chi_f \right) \cdot N_{nano} \cdot V_{nano} + (\chi_{\text{sphere}} - \chi_f) \cdot V_{\text{sphere}} \right] \cdot \nabla \left( \frac{B^2}{2\mu_0} \right)
\]  
(11)

and

\[
(m - m_0) \cdot \eta = \frac{M_{s,nano} \left( \frac{\mu_0}{B} \right) \cdot N_{nano} \cdot V_{nano}}{3\pi d_{\text{sphere}}} - \frac{N_{nano} \cdot V_{nano} \cdot \chi_f}{3\pi d_{\text{sphere}}}  
\]  
(12)

Several further points should be noted with respect to these last three relationships. First, within the analysis region of the CTV instrument, an average value of \( B \) can be assumed with a magnitude of 1.27 T. The field interaction parameter now becomes:

\[
\phi = \left( \frac{M_{s,nano} \mu_0}{B} - \chi_f \right) V_{nano}
\]  
(13).

According to equation (12), a plot of \((m - m_0)\eta\) as a function of \(\chi_f\), predicts that a linear relationship would be obtained. With this modification of \( N \), it follows that the quotient of the y-intercept and slope is \( \phi = \frac{M_{s,nano} \mu_0}{B} \). It should be realized that if a significantly lower magnetic field is used instead (below the level of saturation), Equation 12 does not need to be modified. In our continuing development of electromagnetic CTV system, the magnetic field can be easily adjusted to satisfy the assumption made for equation (1).
In this study, through the use of Equation 9 and or 12, the experimental instrument, Cell Tracking Velocimetry, and buffers of different magnetic susceptibility, the magnetic susceptibility of a number of magnetic nanoparticle. These measurements, multiplied by the volume of the nanoparticles, allows the field interaction parameter to be determined. Finally, the effect that two nanoparticles, of significantly different field interaction parameters, have on magnetically labeled human, peripheral blood lymphocytes will be demonstrated.

3.4 Materials and Methods

3.4.1 Biotinylated microspheres

ProActive® biotin-coated microspheres (catalog number CP10N; Bangs Laboratories, IN, USA) are reportedly uniformly sized polystyrene microspheres, 5.12 µm in diameter, with a standard deviation of 0.1 µm. These microspheres come suspended in 100mM Borate (pH 8.15), 0.1% BSA, 0.05% Tween 20, 10mM EDTA and 0.1% NaN₃.

3.4.2 Preparation of human peripheral blood lymphocytes

Peripheral blood lymphocytes, PBL, were obtained by performing a Ficoll density separation (Accurate Chemical and Scientific Corp., Westbury, NY) on buffy coat purchased from Red Cross and carefully aspirating the mononuclear cell layer formed after centrifugation. The lymphocytes were cultured in medium, RPMI-1640 (ATCC, Manassas, VA), and 10% Fetal bovine serum (FBS; JRH Biosciences, Lenexa, Kansas), at 37°C, 5% CO₂ for two days to deplete adherent monocytes. Mouse anti-human CD3 antibodies (clone # HIT3a) conjugated to phycoerythrin (PE) (BD Biosciences
Phamingen, San Diego, CA) were used to label PBL. Anti PE MACS™ beads (Miltenyi
Biotec, Bergisch Gladbach, Germany) or anti PE magnetic beads (BD Biosciences
Phamingen, San Diego, CA) were then used. Both magnetic particles were used at the
saturation amount.

3.4.3 Magnetic nano particles

Four types of commercial, magnetic nano particles were studied: streptavidin
MACS™ beads (Catalog number 481-01/02; Miltenyi Biotec, CA, USA), BD™ Imag
streptavidin beads (Catalog number 551308, BD Pharmingen, CA, USA), Captivate™
ferrofluid (Catlog number C-21476; Molecular Probes, Eugene, OR) and EasySep™
bears (StemCell Technologies, Canada). All four magnetic nano particles are reported
by their manufacturers to be in the range of 50 to 200 nanometers in diameter. All four
particles consist of an iron oxide core coated with either a polysaccharide or hydrophilic
protein. Streptavidin is coated on the outer surface of the first three particles. The
EasySep™ beads are provided together with bispecific tetrameric antibody complexes
(TAC) in the kit. These complexes recognize both biotin molecule and dextran, a surface
component of the nano particles. Streptavidin MACS™ beads are supplied in a
suspension of 0.1% gelatin with 0.05% NaN₃, and the BD™ Imag streptavidin beads are
suspended in aqueous buffered solution containing 0.09% NaN₃, 0.5% BSA and 2mM
EDTA. The Captivate™ nano particles are supplied with 0.5% BSA and 0.05% ProClin®
300, while the EasySep™ magnetic nano particles are suspended in water.
3.4.4 Method to vary the magnetic susceptibility of the suspending fluid

The magnetic susceptibility of the suspending fluid was varied by the addition of a paramagnetic salt, specifically, Optimark® 0.5mmol/mL (Mallinckrodt Inc, St. Louis, MO). Optimark® solution is sold as a contrast agent for MRI imaging, and consists of gadoversetamide, which is a stable complex formed between a chelating agent and the paramagnetic ion, gadolinium, Gd³⁺, and does not bind to proteins in vitro. Different dilutions of Optimark® in labeling buffer, Dulbecco’s phosphate buffered saline (DPBS modified, JRH Biosciences, Lenexa, Kansas), 2mM ethylene-diamine tetraacetic acid (EDTA, Sigma, St. Louis, MO) were used as suspending fluids in the study.

3.4.5 Measurement of the size of magnetic nanospheres

Two types of experimental instruments were used to measure the size of the nanoparticles: a 90 Plus Submicron Particle Size Analyzer (Brookhaven Instruments Corp., NY, USA), and a MiniDAWN® tri-angle detector (Wyatt Technology, SB, California). The 90 Plus Submicron PSA measures the velocity distribution of particle movement by measuring dynamic fluctuations of the intensity of scattered light, and the particle size is calculated based on the Stokes-Einstein equation. It assumes that particles are spherical and no significant particle-particle interaction is involved.

The MiniDAWN® tri-angle detector uses the technique of classical light scattering (also known as “static” or “Rayleigh” scattering). This method, which reports a number-average mean square radius, involves measuring the amount of light scattered by a solution at some angle relative to the incident laser beam.
3.4.6 Determination of the Physical Properties of the Suspending Fluids

Theoretically, the volumetric magnetic susceptibility (in S.I. unit system) of the suspending fluid can be predicted from:

\[
\chi = \frac{4\pi \cdot [(\text{buffer}) \cdot \chi_{M, \text{buffer}} + [\text{Gd}^{3+}] \cdot \chi_{M, \text{Gd}^{3+}}]}{1000 \text{cm}^3 / L}
\]

\[
= \chi_{\text{buffer}} + \frac{4\pi}{1000 \text{cm}^3 / L} [\text{Gd}^{3+}] \cdot \chi_{M, \text{Gd}^{3+}}
\]  

(14)

where \(\chi_{M, \text{buffer}}\) and \(\chi_{M, \text{Gd}^{3+}}\) are the molar magnetic susceptibility (in CGS unit system) of buffer and gadolinium salt, and [buffer] and [Gd\(^{3+}\)] are molar concentration of buffer and gadolinium salt in the suspending fluid, respectively. From [Weast, 1979], the molar magnetic susceptibility of gadolinium (III) chloride is \(2.7 \times 10^{-2}\) cm\(^3\)mol\(^{-1}\). The conversion factor of 1000cm\(^3\)/L and \(4\pi\) are used to convert CGS unit to SI unit.

Viscosity measurements of these suspending fluids were made on a RFS II Rheometer (Rheometrics, USA) at different temperatures.

3.4.7 Magnetophoretic Mobility measurements

Magnetophoretic mobility measurements were made on an instrument referred to as a Cell Tracking Velocimetry, CTV, discussed previously [McCloskey, 2001; Nakamura, 2001]. Briefly, CTV measures the magnetically induced movement of cells or particles, on a cell-by-cell basis, in a well-defined magnetic field. As stated previously, the value of B varies from 1.38 to 1.15 T in the viewing region. However, the gradient of B varies in an opposite manner such that the Magnetic energy gradient, \(S_m\) (149 T·A/mm\(^2\)), remains nearly constant in the area where the measurement is made (a rectangular viewing area of 1.66mm×1.23mm). Movement of cells or particles in this
area is captured and recorded using an inverted microscope and a 30Hz Cohu CCD 4915 camera (Cohu Electronics, San Diego, CA). The images are digitized into a two dimensional matrix of 640 by 480 pixels by μTech imaging grabbing board (μTech Corp., Billerica, MA). The CTV algorithm, modified from Particle Tracking Velocimetry, PTV, algorithm [Guezennec, 1994], uses five consecutive frames to generate most probable paths of cells or particles. The displacements, more accurately, the number of pixels traveled by the cells or particles in a certain time range are the direct measurement from CTV. Magnetophoretic mobility is obtained with the calibration of dimensions and magnetic field. As a result of continued improvements, this system is semi-automated and an analysis of over 1,000 cells or particles can be performed within 20 minutes.

3.4.8 Preparation of Samples

Seven million biotin-coated microspheres were suspended with specific amounts of magnetic labeling agents in labeling buffer to achieve a final volume of 700 μL. The labeling solution was incubated in an ice bucket with shaking for 30 minutes. Subsequently, 2 mL of buffer was added and the suspension was centrifuged at 500×g for 8 minutes twice. The pellets were resuspended in 350 μL of labeling buffer. 50 μL of the sample was taken out and centrifuged to get rid of as much remaining buffer as possible. The resulted pellet was resuspended with 2mL suspending fluid to achieve a microsphere concentration of 0.5 million/mL for each CTV analysis. The only difference is for EasySep™ magnetic nanoparticles, the biotin-coated microspheres were first labeled with Biotin selection cocktail, and secondly with the nano particles.
3.5 Results

3.5.1 Measurement of the size of the magnetic nano particles

Figure 1 contains representative size distributions of each of the four nano particles, as determined by the 90 Plus Submicron Particle Size Analyzer. Table 2 presents the results in a tabulated form as well as the results for different lot numbers for the EasySep™, and MACS™ nano particles. Table 3 presents the results obtained by using the MiniDAWN® tri-angle detector. As can be observed, there are significant differences in size measurements between the two instruments. While the sizes of BD™ Imag, Captivate™ Ferrofluid and EasySep™ nano particles are approximately the size reported by the manufacturer, there is a batch-to-batch variation in size for MACS™ nano particles, and the size of one batch differs from what the manufacturer claims. Personal communications with some of the manufactures indicate that nano particles synthesized usually go through several filtration steps to control nanoparticle size. Such filtering is probably the cause of the sharp cut-offs observed in Figure 1.

The size of the ProActive® biotin-coated microspheres and magnetic microspheres were measured with a Coulter® Multisizer II (Coulter Electronics, FL) and the results are presented in Table 4. For the ProActive® microspheres, the measured mean diameter was close to manufacturers specification; however the standard deviation was significantly higher.

3.5.2 Physical properties of various suspending fluids

Figure 2 is a plot of the volumetric magnetic susceptibility (A) and viscosity (B) of suspending fluid as a function of molar concentration of Gd3+ salt. Experimental
measurements are presented as symbols, with associated error bar of one standard deviation, while the solid line is the magnetic susceptibility predicted by Equation (12).

3.5.3 Magnetic Particle-Particle Interaction

Previous studies [McCloskey, 2001; Comella, 2001] indicated that at cell or particle concentrations on the order of 1 million per mL, or greater, detectable particle-particle interaction, affecting magnetophoretic mobility measurements, begins to be observed. Since fundamental to this study are highly accurate, experimental measurements of magnetophoretic mobility of microspheres, further studies were conducted with 5 micron magnetite/maghemite-coated polymeric microspheres. A complete description and evaluation of these magnetic microspheres has been previously published [Moore, 2000].

Figure 3 presents a histogram of four independent experiments measuring the magnetophoretic mobility of magnetite/maghemite-coated polymeric microspheres at the number concentration of 2, 1, 0.5, and 0.2×10^6 microspheres per mL. As can be observed, and tabulated in Table 5, as the concentration decreases, the mobility decreases and then stabilizes around 1.25×10^{-4} mm^3/T-A-s (or mm^3-s/kg). All the CTV experiments on ProActive® microspheres were thus performed at the number concentration of 0.5×10^6 microspheres per mL.

3.5.4 Magnetic Susceptibility of ProActive® biotin-coated microspheres

To both test the experimental approach to measure the magnetic susceptibility of the microspheres and nano particles (Equations 5, 9 and 12) as well as experimentally determine the magnetic susceptibility of the ProActive® microspheres for later
calculations of the magnetic susceptibility of the various commercial, magnetic nanoparticles, the magnetophoretic mobility of the unlabeled ProActive® microspheres was measured in suspending fluid of varying magnetic susceptibility. Table 6 lists a set of magnetophoretic mobility measurements. Pair-wise t tests support the conclusion that magnetophoretic mobility of ProActive® microspheres differs at different gadolinium concentrations. Representative histograms of several of these studies are presented in Figure 4a and all of the data are plotted in the form of the product of the measured magnetophoretic mobility and suspending solution viscosity versus the volumetric susceptibility of the suspending fluid (Figure 4b).

As can be observed, a linear relationship was obtained with a slope of $-2.52 \times 10^{-3}$ and intercept of $-1.94 \times 10^{-8}$, $R^2 = 0.978$, n=18. As presented previously with Equation (5), the quotient of the intercept and the slope is, $\chi_{sphere}$, which in this case provides a value of $-0.77 \times 10^{-5}$. This value compares with the reported value for styrene of $-0.75 \times 10^{-5}$ [Weast, 1979] and an experimentally measured value for polystyrene of $-0.82 \times 10^{-5}$ [Watarai, 2001].

3.5.5 Magnetic Susceptibility of four commercial nanoparticles

Table 7 lists mobility measured by CTV of labeled polystyrene microspheres (with 2µL streptavidin microbeads). More than 1000 particles are tracked to make the statistically important measurements, and the narrow 95% confidence interval shows the reliability of the measurements. Figures 5-10 are plots, similar to Figure 4b, except in these figures the ProActive® microspheres were labeled with various amounts of commercial, magnetic nanoparticles. Each set of symbols (and regression line associated
with those symbols) corresponds to a set of experiments where the microspheres were labeled with a specific concentration of magnetic nano particles. This suspension was then divided and resuspended into suspending fluids with varying volumetric susceptibility, which is given on the x-axis. $m_o$, the magnetophoretic mobility of unlabeled ProActive® biotin-coated microspheres is the averaged experimental value. Table 8 presents the slope, intercept, quotient of the intercept and slope, and the average of this quotient, for each set of experiments conducted with a given lot of magnetic nano particles. Also, the estimates of number of magnetic nano particles present on the surface of the microsphere and the number of possible layers formed are listed. It should also be noted that these same lots were analyzed previously for size and the results are listed in the previous tables.

3.5.6 Calculation of the particle-field interaction parameter

As presented previously, the product of the difference in the volumetric magnetic susceptibility between the magnetic label (or more generally the particle or cell) and the suspending fluid, and the volume of that magnetic entity has been called the particle-field interaction parameter. In the case of the iron nanoparticles in the CTV instrument, this field interaction parameter is defined by equation (13). The product of this field interaction parameter and the magnetic energy gradient, $S_m$, (Equation 1), determines the magnitude of the magnetic force operating on the entity of interest. Table 9 lists the magnetic nano particles, the specific lot number, the calculated magnetic susceptibility, the measured mean diameter, and the calculated particle-field interaction parameter. It
should be noted that it was assumed that the suspending fluid is composed purely of buffer and that the magnetic nano particle was assumed to be a perfect sphere.

To demonstrate the impact that this difference in field interaction parameter has on immunomagnetically labeled cells, two equally prepared sets of suspensions of human PBL were labeled with four different, increasing amounts of a primary antibody, anti-CD3 PE conjugates. Next, one set of labeled cells were labeled with anti-PE MACS nanoparticles and the other set was labeled with anti-PE-Imag nanoparticles. For each concentration of primary antibody reported in Figure 11 (antiCD-PE), four concentrations of secondary antibody (either anti-PE MACS or anti-PE Imag) were used: 5, 20, 40, and 80µl in 100µl of 10^6 cell suspension. After labeling, the magnetophoretic mobility of these cell suspensions was measured in the CTV instrument and the highest, saturated magnetophoretic mobility obtained at each primary antibody concentration was presented in Figure 11. As can be observed, for the same concentration of primary antibody, the anti-PE-Imag labeled cells had a significantly higher (almost an order of magnitude) magnetophoretic mobility than the cells labeled with the anti-PE-MACS. This difference is consistent with the significantly higher field interaction parameter of the Imag nanoparticles relative to the MACS nanoparticles.

3.6 Discussion

At the most fundamental level, the method used in this manuscript to determine the volumetric susceptibility of the micro and nano particles originated from the “isomagnetic” concept, that is, a magnetically susceptible material will move at a velocity proportional to the difference in the magnetic susceptibility between the entity of interest and the suspending fluid (Equation 3) [Russell, 1987]. More recently [Moore, 2004], this
“isomagnetic” concept was used to improve the resolution between magnetic and non-magnetical particles and the performance of a quadrupole magnetic flow sorter; both improvements were consistent with theoretical calculations using the isomagnetic concept.

The linear result of measured magnetophoretic mobilities of the unlabeled ProActive® polystyrene microspheres in suspending fluids of different magnetic susceptibility (Figure 4b) obtained in this study confirms the overall approach. In addition to the regression of the data (solid line in Figure 4b), the dashed lines represent the 95% confidence interval of $[-1.16 \times 10^{-5}, -0.50 \times 10^{-5}]$. Compared to the reported values of polystyrene magnetic susceptibility of $-0.75 \times 10^{-5}$ and $-0.82 \times 10^{-5}$, this analysis provides confidence in the reliability of this approach.

While not ideal, the plots of the magnetophoretic mobility of the ProActive® microspheres (labeled with different magnetic nanoparticles), in suspending fluids of different magnetic susceptibility (Figures 5 -10), allow reasonable calculations to be made of the saturation magnetic moment of nanoparticles. These measurements, combined with the experimental measurements of the size of the nano particles, allow estimates to be made of the particle-field interaction parameter, $\phi$.

Questions can be raised with respect to the magnitude of $\phi$. First, are the various values of this parameter correct, and second, what is their significance? Since the current, primary interest with magnetic nano particles is cell separation, and as with any analysis or separation technology the ratio of signal to noise greatly influences the quality of the results, a comparison of the magnitude of $\phi$ for various magnetic reagents, and
more generally cells, is in order. Table 10 presents the value of $\phi$, either experimentally determined or theoretically estimated, for several cells as well as ferritin molecules and Dynabeads. These values were obtained from literature data and, in the case of cells, calculated using the following relationship with the published values of $m$:

$$\Delta z V_{cell} = \phi = m \cdot (3\pi\eta d_{cell})$$

(15)

In terms of the accuracy of these values, an independent study on the value of $\phi$ for anti-FITC MACS™ nanoparticles by [McCloskey, 2000] estimated the particle-field interaction parameter to be $2.5 \times 10^{-25}$ m$^3$ which is close to the values of $2.3\sim8.8 \times 10^{-25}$ m$^3$ calculated for the MACS™ reagents presented in this study.

A number of significant observations can be made comparing the values of $\phi$ presented in Tables 9 and 10. First, the magnitude of $\phi$ is clearly a function of the size of the magnetic carrier, ranging over 11 orders of magnitude while the nominal diameter ranges over 400 times (comparing ferritin to Dynabeads). Second, the saturation magnetic moment of the various commercial magnetic nanoparticles (Table 9) is quite similar, indicating for these commercial nano particles the size (volume) is the primary factor determining the value of $\phi$. Third, the value of $\phi$ for most cells, except deoxygenated red blood cells, is negative, confirming the diamagnetic character of cells. This negative value of $\phi$ has a significant implication: unlike the autofluorescence signals that cells typically exhibit, and which can interfere with fluorescence probes used to label cells, there is no “automagnetism” interference. Consequently, it is theoretically possible to measure fewer numbers of antibody-magnetic nano particle conjugates bound to a cell than with antibody-fluorescent probe conjugates. Such studies are currently
ongoing. Fourth, depending on the expression level of the cell marker of interest, a
number of potential magnetic labels are available, i.e. if only a few markers are
expressed, magnetic labels with a high $\phi$ are desirable, and the opposite is true for a high
expressing cell. This effect of the magnitude of the value of $\phi$ on a cell magnetophoretic
mobility is clearly shown in Figure 11. An almost one order of magnitude increase in the
magnetophoretic mobility of immunomagnetically labeled human lymphocytes is
observed when the BD Imag nanoparticle are used compared to the MACSTM nanobeads.
Previous research, both theoretical and experimental, in our laboratory has demonstrated
the improved performance of cell separation in the Quadrupole Magnetic Flow Sorter as
a function of the labeled cells magnetophoretic mobility [Williams, 1999; McCloskey,
2003].

The data plotted in Figures 5 through 10, and the subsequent calculations of the
saturation magnetic moment were based on the relationship expressed in Equations (12
and 13). As can be noted by further examination of Equation 12 the diameter of the
labeled microsphere, $d_{\text{sphere}}$, appears in the slope and intercept, yet cancels in the
calculation of the saturation magnetic moment. However, the number of magnetic nano
particles bound to the microsphere can be calculated from the slope of the curve and this
number is presented in Table 8. If one assumes a monolayer binding, one can calculate
the number of layers of the specific nanobead on the microbeads (relationship used is
presented in the Appendix) and the numbers of layers is also presented in Table 8, using
two different experimentally measured nanoparticle diameters. Inspection of Table 8
indicates that significant multi layer coverage is predicted for the CaptivateTM and
EasySep™ nanoparticles. This conclusion is in agreement with both public and private conversations with particle manufacturers. These calculations also indicated the apparent sensitivity of the number of bound nanoparticles to the nanoparticle size.

To estimate the effect that either a monolayer, or multilayer covering will have on calculations using Equation 12, or other related Equations, Figure 12 presents the relative change in the friction factor as a function of layers of different diameter nanobeads on the 5.12 µm polystyrene microsphere. Clearly, a monolayer of any size of the nanobeads has a minimal effect on drag, and subsequent mobility. However, when multilayer binding of nanobeads occurs, or when large nanobeads (>150 nm) are used, three to four layers can create at least a 10 percent increase in the drag potentially impacting analysis of magnetophoretic mobility measurements.

Further comments with respect to the accuracy of the calculations are in order. First, the magnetic nanoparticles most likely do not perform as rigid spheres, and the number of particles calculated for a monolayer is only an estimate. Second, when high concentrations of paramagnetic solutions, such as gadolinium salts, were used to suspend the microspheres, initially circulation patterns were observed. Consequently, images were taken after the circulation became neglectable, thus some fast moving microspheres were not recorded and the reported mobility underestimates the true mean magnetophoretic mobility. If these two possibilities are considered, the number of magnetic particles bound onto the microsphere surface should be fewer than what are shown in the tables.
In conclusion, this study presents a methodology which can be used to measure the magnetic susceptibility of magnetic micro and nano particles. These measurements allow both a better understanding of the fundamentals of making a cell magnetic as well as provide insight to the choice of the best nano particles for a particular application.

3.7 Acknowledgements

This work has been supported by the National Science Foundation (BES-9731059 to J.J.C. and CTS-0125657 to P.S.W.) and the National Cancer Institute (R01 CA62349 to M.Z. and R33 CA81662-01 and R01 CA097391 to J.J.C.).

3.8 Note

As shown in the left, three perfect spheres are clustered on the surface of a microsphere. It can be shown that one sphere covers \( \frac{\sqrt{3}}{4} d^2 \), where \( d \) is the diameter of the sphere. For this relationship, the number of perfect spheres covered on the surface of a larger, perfect sphere is calculated.
3.9 Nomenclature

\( F \) = Force (N)

\( B \) = Magnetic field induction (T)

\( d \) = Diameter (m)

\( m \) = Magnetophoretic mobility (\( \text{mm}^3\cdot\text{s/kg or mm}^3/\text{T-A-s} \))

\( M_{s,\text{nano}} \) = saturation magnetic moment of the nano particles

\( S_m \) = Magnetic energy gradient (\( T \cdot A/m^2 \))

\( V \) = Volume (m\(^3\))

**Greek**

\( \phi \) = Particle-field interaction parameter (m\(^3\))

\( \chi \) = Volumetric magnetic susceptibility, SI units used (-)

\( \eta \) = Viscosity (kg/m-s)

\( \mu_0 \) = Magnetic permeability of free space (N/A\(^2\))

**Subscripts**

\( \mu_{\text{sphere}} \) = Microsphere

\( \text{nano} \) = Nano particles

\( f \) = Fluid
3.10 Reference


### Table 3.1 Volumetric magnetic susceptibilities of specific elements and compounds (in SI unit system)

<table>
<thead>
<tr>
<th>Substance</th>
<th>Formula</th>
<th>$\chi$ ($\times 10^{-6}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>H$_2$O</td>
<td>-9.05</td>
<td>Zborowski, 1995</td>
</tr>
<tr>
<td>Carbon (diamond)</td>
<td>C</td>
<td>-252.8</td>
<td>Weast, 1979</td>
</tr>
<tr>
<td>Carbon (graphite)</td>
<td>C</td>
<td>-418.9</td>
<td>Weast, 1979</td>
</tr>
<tr>
<td>Styrene</td>
<td>C$_8$H$_8$</td>
<td>-7.46</td>
<td>Weast, 1979</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>C$<em>6$H$</em>{12}$O$_6$</td>
<td>-10.92</td>
<td>Weast, 1979</td>
</tr>
<tr>
<td>Erbium oxide</td>
<td>Er$_2$O$_3$</td>
<td>20981</td>
<td>Weast, 1979</td>
</tr>
<tr>
<td>Iron (III) oxide (hematite)</td>
<td>$\alpha$-Fe$_2$O$_3$</td>
<td>500–40,000</td>
<td>Hunt, 1995</td>
</tr>
<tr>
<td>Iron (III) oxide (maghemite)</td>
<td>$\gamma$-Fe$_2$O$_3$</td>
<td>2,000,000–2,500,000</td>
<td>Hunt, 1995</td>
</tr>
<tr>
<td>Iron (II) oxide (magnetite)</td>
<td>Fe$_3$O$_4$</td>
<td>1,000,000–5,700,000</td>
<td>Hunt, 1995</td>
</tr>
</tbody>
</table>

### Table 3.2 Size of magnetic nanoparticles measured by photon correlation spectroscopy (PCS). The mean diameter and standard deviation is based on the result of size distribution of several hundred nanoparticles.

<table>
<thead>
<tr>
<th>Magnetic Nanobeads</th>
<th>Lot #</th>
<th>Mean Diameter (nm)</th>
<th>Standard Deviation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EasySep™</td>
<td>3A317176</td>
<td>160</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>2L226593</td>
<td>142</td>
<td>8</td>
</tr>
<tr>
<td>Captivate™</td>
<td>71A1-1</td>
<td>136</td>
<td>15</td>
</tr>
<tr>
<td>BD™ Imag</td>
<td>44023</td>
<td>231</td>
<td>23</td>
</tr>
<tr>
<td>Streptavidin-MACS™</td>
<td>5020918049</td>
<td>67.2</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>5020305031</td>
<td>116</td>
<td>1</td>
</tr>
<tr>
<td>Basic MACS™</td>
<td>5010814011</td>
<td>107</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>5020813004</td>
<td>127</td>
<td>1</td>
</tr>
<tr>
<td>Anti-FITC-MACS™</td>
<td>5000403063</td>
<td>61.9</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>5990823042</td>
<td>67.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Magnetic Carriers</td>
<td>Mean Diameter (nm)</td>
<td>Standard Deviation (nm)</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>--------------------</td>
<td>-------------------------</td>
<td></td>
</tr>
<tr>
<td>MACSTM beads</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Streptavidin Lot # 5020918049</td>
<td>130</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Lot # 5020305031</td>
<td>180</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Basic Lot # 5010814011</td>
<td>70</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Captivate™ (Lot # 71A1-1)</td>
<td>180</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3 Size of magnetic nanoparticles measured using the MiniDAWN® triangle detector. The mean diameter and standard deviation is based on the size distribution for several hundred nanoparticles.

<table>
<thead>
<tr>
<th>Micro Particles</th>
<th>Mean Diameter (µm)</th>
<th>Standard Deviation (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProActive® Biotin-coated microspheres</td>
<td>5.12</td>
<td>0.56</td>
</tr>
<tr>
<td>Magnetic microspheres</td>
<td>4.85</td>
<td>0.95</td>
</tr>
</tbody>
</table>

Table 3.4 Size of ProActive Biotin-coated microspheres using a Coulter counter. The mean diameter and standard deviation is based on the results of size distribution of several hundred microspheres.

<table>
<thead>
<tr>
<th>Bead Type</th>
<th>Particle Concentration (million/mL)</th>
<th>Volume Percentage (%)</th>
<th>Mean Mobility (mm³-s/kg)</th>
<th>95% Confidence Interval (mm³-s/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Magnetic microspheres</td>
<td>0.2</td>
<td>0.0012</td>
<td>1.2E-04</td>
<td>[1.183E-04, 1.217E-04]</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.0030</td>
<td>1.3E-04</td>
<td>[1.287E-04, 1.313E-04]</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.0060</td>
<td>1.4E-04</td>
<td>[1.389E-04, 1.411E-04]</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.0119</td>
<td>1.8E-04</td>
<td>[1.789E-04, 1.811E-04]</td>
</tr>
</tbody>
</table>

Table 3.5 Magnetophoretic mobility value of magnetic microspheres measured under different microsphere concentration in buffer (PBS, 2mM EDTA)
### Table 3.6 CTV results of unlabeled polystyrene microspheres in varying concentrations of gadolinium solution.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>[Gd(^{3+})] (M)</th>
<th># of Particles</th>
<th>Mean Magnetophoretic Mobility (mm(^3)-s/kg)</th>
<th>Standard Deviation of Mobility (mm(^3)-s/kg)</th>
<th>95% C.I. of mean (mm(^3)-s/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0000</td>
<td>770</td>
<td>3.07E-06</td>
<td>4.67E-06</td>
<td>[2.74E-06, 3.40E-06]</td>
</tr>
<tr>
<td>2</td>
<td>0.0200</td>
<td>1335</td>
<td>-1.57E-05</td>
<td>2.13E-05</td>
<td>[-1.69E-05, -1.46E-05]</td>
</tr>
<tr>
<td>3</td>
<td>0.0500</td>
<td>911</td>
<td>-3.21E-05</td>
<td>1.62E-05</td>
<td>[-3.32E-05, -3.11E-05]</td>
</tr>
<tr>
<td>4</td>
<td>0.0625</td>
<td>872</td>
<td>-4.54E-05</td>
<td>2.66E-05</td>
<td>[-4.72E-05, -4.36E-05]</td>
</tr>
<tr>
<td>5</td>
<td>0.1000</td>
<td>928</td>
<td>-4.94E-05</td>
<td>2.91E-05</td>
<td>[-5.13E-05, -4.75E-05]</td>
</tr>
<tr>
<td>6</td>
<td>0.1667</td>
<td>1005</td>
<td>-8.47E-05</td>
<td>2.42E-05</td>
<td>[-8.62E-05, -8.32E-05]</td>
</tr>
</tbody>
</table>

### Table 3.7 CTV results of polystyrene microspheres labeled with 2µL streptavidin-MACS™ (Lot # 5020918049) in varying concentrations of gadolinium solution.

<table>
<thead>
<tr>
<th>[Gd(^{3+})] (M)</th>
<th>Mean Magnetophoretic Mobility (mm(^3)-s/kg)</th>
<th>Standard Deviation of Mobility (mm(^3)-s/kg)</th>
<th># of Particles</th>
<th>95% C.I. of mean (mm(^3)-s/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0000</td>
<td>8.44E-05</td>
<td>1.93E-05</td>
<td>1463</td>
<td>[8.34E-05, 8.53E-05]</td>
</tr>
<tr>
<td>0.0200</td>
<td>5.86E-05</td>
<td>2.08E-05</td>
<td>1735</td>
<td>[5.76E-05, 5.96E-05]</td>
</tr>
<tr>
<td>0.0500</td>
<td>3.98E-05</td>
<td>1.76E-05</td>
<td>1508</td>
<td>[3.89E-05, 4.07E-05]</td>
</tr>
<tr>
<td>0.0625</td>
<td>2.21E-05</td>
<td>2.13E-05</td>
<td>1458</td>
<td>[2.10E-05, 2.32E-05]</td>
</tr>
<tr>
<td>0.1667</td>
<td>-2.54E-05</td>
<td>1.91E-05</td>
<td>1002</td>
<td>[-2.66E-05, -2.42E-05]</td>
</tr>
</tbody>
</table>
Table 3.8 Numeric values of the intercept, slopes, and quotient of the data presented in Figures 3.5-10. As predicted by equation (11), for the same kind of magnetic nanobeads, the intercept and the absolute value of slope become higher with the higher mobility. The ratio of intercept and the absolute value of slope should remain constant for each magnetic particles as indicated by equation (11).

*, calculation of number of nano particles bound onto microsphere surface is based on Equation (10).

**, calculation of number of nano particles bound onto microsphere surface in monolayer is shown in Appendix using diameter of nanoparticle based on PCS measurements.

***, calculation of number of nano particles bound onto microsphere surface in monolayer is shown in Appendix using diameter of nanoparticle based on MiniDAWN tri-angle measurements.
<table>
<thead>
<tr>
<th>Magnetic Beads</th>
<th>Lot #</th>
<th>$M_{s,nano}$ /B (SI unit system)</th>
<th>Mean Diameter (nm)</th>
<th>$\phi$ ($\times10^{-25} m^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptavidin-MACS</td>
<td>5020305031</td>
<td>1.1 ±0.3 E-03</td>
<td>116</td>
<td>8.8 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>5020918049</td>
<td>1.4 ± 0.4 E-03</td>
<td>67.2</td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td>BD Imag</td>
<td>44023</td>
<td>1.4 ± 0.3 E-03</td>
<td>231</td>
<td>91 ± 20</td>
</tr>
<tr>
<td>Captivate</td>
<td>71A1-1</td>
<td>3.8 ± 1.0 E-04</td>
<td>136</td>
<td>5.1 ± 1.4</td>
</tr>
<tr>
<td>EasySep</td>
<td>2L226593</td>
<td>3.5 ±1.2 E-04</td>
<td>142</td>
<td>5.4 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>3A317176</td>
<td>5.5 ±1.8 E-04</td>
<td>160</td>
<td>12 ± 4</td>
</tr>
</tbody>
</table>

Table 3.9 For specific lot numbers, the magnetic susceptibility, the mean diameter, and the particle-field interaction parameter, $\phi$, for the four types of magnetic nanobeads studied. To calculate $\phi$, the volume of the nanoparticle was assumed to be a perfect sphere using the experimentally measured mean diameter, and suspending fluid has a magnetic susceptibility of water.

<table>
<thead>
<tr>
<th>Particle/Cell</th>
<th>Estimated Diameter (nm)</th>
<th>Estimated Volume (m$^3$)</th>
<th>$\phi$ (m$^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Theoretical Estimation</td>
</tr>
<tr>
<td>Lymphocyte</td>
<td>7,100</td>
<td>$1.8 \times 10^{-16}$ a</td>
<td>-</td>
</tr>
<tr>
<td>Human RBC (oxygenated)</td>
<td>7,700 c (hydrodynamic)</td>
<td>$8.84 \times 10^{-17}$ c</td>
<td>$-1.6 \times 10^{-23}$</td>
</tr>
<tr>
<td>Human RBC (deoxygenated)</td>
<td>7,700 c (hydrodynamic)</td>
<td>$8.84 \times 10^{-17}$ c</td>
<td>$3.4 \times 10^{-22}$</td>
</tr>
<tr>
<td>Ferritin (native)</td>
<td>12</td>
<td>$9 \times 10^{-25}$ d</td>
<td>$9.5 \times 10^{-29}$ d</td>
</tr>
<tr>
<td>Ferritin (magnetoferritin)</td>
<td>&quot;</td>
<td>&quot;</td>
<td>$7.8 \times 10^{-28}$ d</td>
</tr>
<tr>
<td>Dynabead</td>
<td>4,500</td>
<td>$4.77 \times 10^{-17}$</td>
<td>$1.15 \times 10^{-17}$ a</td>
</tr>
</tbody>
</table>

Table 3.10 Reported and experimentally calculated, values of the particle-magnetic field interaction parameter, $\phi$, ($m^3$).

a Data from [McCloskey, 2001] and assuming a perfect sphere.

b Calculated using Equation (11), and a reported magnetophoretic mobility of $-5.2 \times 10^{-6}$ mm$^3$/kg. Data from [McCloskey, 2001].

c Data from [Zborowski, 2003].

d Data from [Zborowski, 1995].

e Data from [Reddy, 1996].
Figure Legends

Figure 3.1 Histograms of the size distribution of Streptavidin MACSTM nanobeads (a), BD™ Imag streptavidin nanobeads (b), EasySep nanobeads (c) and Captivate™ Ferrofluid (d) measured using a 90 Plus Submicron Particle Size Analyzer.

Figure 3.2 A plot of the volumetric susceptibility (A) and viscosity (B) of Optimark® dilutions as a function of Gd$^{3+}$ concentration. The volumetric susceptibility were estimated based on Equation (12), and the viscosity measurements were made on a RFS II rheometer (Rheometrics).

Figure 3.3 A histogram of the experimentally measured magnetophoretic mobility of the magnetic polymeric microspheres at different concentrations. The magnetophoretic mobility was experimentally measured on a Cell Tracking Velocimetry instrument.

Figure 3.4 A histogram of experimentally measured magnetophoretic mobility of ProActive microspheres (5.2 microns) in suspending fluids of different magnetic susceptibility (a) and a scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) in different suspending fluids. The linear line was the regression obtained following equation (5).

Figure 3.5 A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of streptavidin-MACS™ beads (Lot # 5020918049) in different suspending fluids.

Figure 3.6 A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of streptavidin-MACS™ beads (Lot # 5020305031) in different suspending fluids.

Figure 3.7 A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of EasySep™ beads (Lot # 2L226593) in different suspending fluids.

Figure 3.8 A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of EasySep™ beads (Lot # 3A317176) in different suspending fluids.
Figure 3.9 A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of BD™ Imag streptavidin nanobeads (Lot # 0000044023) in different suspending fluids.

Figure 3.10 A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of Captivate™ beads (Lot # 71A1-1) in different suspending fluids.

Figure 3.11 A comparison of magnetophoretic mobility for T cells labeled with anti CD3 PE and two anti PE magnetic nano particles.

Figure 3.12 Influence of multiple layers formed after labeling on friction factor on the labeled microspheres.
Figure 3.1a-d Histograms of the size distribution of Streptavidin MACS™ nanobeads (a), BD™ Imag streptavidin nanobeads (b), EasySep streptavidin beads (c) and Captivate™ Ferrofluid (d) measured using a 90 Plus Submicron Particle Size Analyzer.
Figure 3.2A

Figure 3.2B

Figure 3.2  A plot of the volumetric susceptibility (A) and viscosity (B) of Optimark® dilutions as a function of Gd$^{3+}$ concentration. The volumetric susceptibility was estimated based on Equation (12), and the viscosity measurements were made on a RFS II rheometer (Rheometrics).
Figure 3.3 A histogram of the experimentally measured magnetophoretic mobility of the magnetic polymeric microspheres at different concentrations. The magnetophoretic mobility was experimentally measured on a Cell Tracking Velocimetry instrument.
Figure 3.4 A histogram of experimentally measured magnetophoretic mobility of ProActive microspheres (5.2 microns) in suspending fluids of different magnetic susceptibility (a), and a scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) in different suspending fluids. The linear line was the regression obtained following equation (5) (b).
Figure 3.5 A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of streptavidin-MACS™ beads (Lot # 5020918049) in different suspending fluids.
Figure 3.6  A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of streptavidin-MACS™ beads (Lot # 5020305031) in different suspending fluids.
Figure 3.7 A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of EasySep™ beads (Lot # 2L226593) in different suspending fluids.
Figure 3.8 A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of EasySep™ beads (Lot # 3A317176) in different suspending fluids.
Figure 3.9 A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of BD™ Imag streptavidin nanobeads (Lot # 0000044023) in different suspending fluids.
Figure 3.10  A scatter plot of experimentally measured magnetophoretic mobility of biotin-coated polystyrene microspheres (5.2 microns) labeled with different amount of Captivate™ beads (Lot # 71A1-1) in different suspending fluids.
Figure 3.11  A comparison of magnetophoretic mobility for T cells labeled with anti CD3 PE and two anti PE magnetic nano particles.
Figure 3.12  Influence of multiple layers formed after labeling on friction factor on the labeled microspheres.
CHAPTER 4: THE EFFECT OF MAGNETIC NANO PARTICLES ON THE BINDING AFFINITIES/AVIDITY OF ANTIBODIES-ANTIGEN AND STREPAVIDIN-BIOTIN INTERACTIONS

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4.1 Abstract

It has long been found that the magnetophoretic mobility of labeled cells shows a saturation type phenomenon with the increase in the amount of labels used. Typical antibody antigen interaction model was used to describe the interaction happened in a homogeneous environment, that is in a solution. To describe the binding of antibody to antigenic determinant on the cell surface (heterogeneous binding), a binding model was proposed in this paper. The binding of anti CD3 antibody FITC, PE, and DM (magnetic beads) conjugates to the enriched peripheral blood lymphocytes was studied with the use of flow cytometry, and CTV. The obtained data was fitted using the proposed data. It was found that the sterric hindrance resulted from the increase in conjugate size affect both the binding affinity and the antibody binding capacity.

A scale up model was then proposed so that the parameters obtained from a small scale experiments can be used to predict the outcome of large scale immunological labeling, which has important applications as labeling of peripheral blood lymphocytes with anti CD3 antibody at large scale for depletion is an important step in bone marrow transplantation. Results obtained from the labeling of up to 40 million cells were compared with that predicted from the model to check the validity of the model.
4.2 Introduction

As is well known, bioaffinity interactions exist widely in nature, and engineering applications of these interactions for use in separations and detections range widely from the use in affinity chromatography for separation of commercial products in bioprocessing to detection technology for human diagnostics to the most recent applications in biowarfare detection [Polanowski, 2003; Hiss, 2003; Safar, 2002]. A fundamental measure of the performance of a bioaffinity interaction is the strength of the noncovalent interactions between a single antibody-antigen (or two molecules in general, i.e. biotin-avidin), referred to as the affinity of the interaction [Goldsby, 2000]. When antibodies are used to bind to cells, typically, complex antigens are present with multiple repeating antigenic determinates resulting in multiple binding sites for the antibody. The presence of these multiple binding sites can have a cooperative effect on the binding of the antibody to the cell. In addition, typically used immunoglobulin G (IgG) is a dimer, each with one binding site for the antigenic determinants. Consequently, the strength of such multiple interactions is referred to as avidity of the antibody-antigen interaction.

The binding constants of these bioaffinity interactions fundamentally define the performance of affinity based separation and diagnostic/detection systems and modifications in the magnitude of these parameters can have profound effects on the performance and economics of the technology. Over the years, a number of entities have been used to covalently bind to the antibodies and/or affinity molecule to assist in the detection of its binding. These entities, commonly referred to as tags, range from radioactive atoms, fluorescent molecules, gold nanoparticles, to magnetic nano and
microparticles [Bobrovnik, 2001; Benedict, 1997; Friguet, 1993]. Magnetic particles are typically made of magnetically susceptible materials embedded in a polymer material [Safarik, 1999]. Despite the basically similar structure, the commercially available magnetic particles differ significantly in size: from the size of a cell, on the order of 5 microns (i.e. Dyna M-500 beads, Dynal AS, Oslo, Norway), to a size not much bigger than an antibody, on the order of 50 nanometers, (MACSTM beads, Miltenyi Biotec, Bergisch Gladbach, Germany).

While magnetic micron sized particles have been in use for over 20 years, the more recent advent of magnetic nanoparticles, one of the more popular is the 50nm MACSTM beads (Mylteni Biotech), have significantly increased the popularity of immunologically based magnetic separations [Thiel, 1998; Bucak, 2003; Despres, 2000; Hu, 2003]. A number of advantages exist with the use of magnetic nanoparticles over magnetic microparticles, not the least of which is the colloidal behavior of antibodies, or affinity tags bound to magnetic nanoparticles. While the very small nature of the magnetic nanobeads requires a significant number of antibody-nanoparticle conjugates to be bound before a separation can be accomplished (unlike the binding of a single, magnetic microbead), it has been demonstrated that over specific ranges, the magnetic force that is imparted on cells labeled with these antibody-nanoparticle conjugates (when the cells are placed in a magnetic energy gradient) is proportional to the antibody binding capacity, $ABC$, of the cell [McCloskey, 2000].

This experimentally observed proportionality of the imparted magnetic force, reported using the term magnetophoretic mobility, $m$, and the cells’ ABC, presents the
possibility of separating cells not just in a binary mode of operation but also based on the surface expression level of the cell [Chalmers, 1998; McCloskey, 2003]. In addition, it was observed that the magnetophoretic mobility of immunomagnetically labeled cells is a saturation type function of the concentration of the antibody-nanobead conjugates used to label the cells [Chosy, 2003; Comella, 2001]. While a saturation type relationship is theoretically expected, based on typical antibody-antigen interactions, when immunomagnetic cell separations are scaled-up to clinical applications, the amount of antibody needed is financially non-trivial.

This manuscript focuses on the manipulation of theoretical relationships describing both the binding affinity and operation of analytical instruments, combined with experimental studies using these instruments, to quantify the binding constants of antibodies-antigen and biotin-streptavidin interactions when one of the binding entities is covalently bound to a magnetic nanoparticle.

4.3 Theory

If one assumes monovalent binding, at equilibrium, the interaction of a receptor-ligand complex can be expressed by:

\[
R + L \overset{k_a}{\underset{k_d}{\rightleftharpoons}} RL
\]

where \(k_a\) and \(k_d\) are the rate constants for the association and dissociation reaction, and \([R], [L],\) and \([RL]\) are the concentrations of free receptor, free ligands, and receptor-ligand complexes, respectively. Two constants are widely used to characterize and/or
express the strength of this interaction: an equilibrium dissociation constant, $K_D$, and an equilibrium association constant, $K_A$, given by equation (2):

$$K_D = \frac{k_d}{k_a} = \frac{[R][L]}{[RL]} = \frac{1}{K_A} \tag{2}$$

It is generally reported that $K_D$ is a quantitative indicator of the stability of receptor-ligand interactions, with low values representing stable (strong) interactions and high values representing weak interactions. Table 1 presents the range of $K_D$ for a number of affinity interactions [Garcia, 1999]. It should be noted that the dissociation constant, $K_D$, defined in equation (2), is based on the concept of thermodynamic equilibrium for species suspended in a homogeneous solution [Goldberg, 1993].

The classical Scatchard analysis is based on this equilibrium assumption and the experimental results from a typical dialysis experiment [Goldsby, 2000] can be used to determine the dissociation constant using the following relationship:

$$\frac{r}{[R]} = \frac{1}{K_D} (n - r) \tag{3}$$

where

$$r \equiv \frac{[R]_{Total} - [R]}{[L]_{Total}} \tag{4}$$

is a ratio of the concentration of ligands bound with receptors to the total concentration of ligands, $[R]$ is the free receptor concentration at equilibrium, and $n$ is the valence of the ligand binding, or the number of receptors one ligand binds.
Currently, there is no straightforward thermodynamic theory available to describe the binding equilibrium in heterogeneous phase systems, such as the binding of an antibody to the antigen on cell surface or on an ELISA plate. Therefore, the binding constants obtained in such systems should be called “apparent binding constant”, which may not have values anywhere close to the true binding constants obtained in suspension. This apparent binding constant takes into consideration potential antibody valence, steric hindrance, or other non-ideal affects [Goldberg, 1993]. In order to determine this apparent binding constant, the following model, and experimental analysis, was constructed and conducted.

**Binding Model** Taking the antibody antigen interaction as an example, when an antibody-conjugate binds to a cell, at least four scenarios can happen: A) monovalent binding, B) homogeneous bivalent binding, C) heterogeneous bivalent binding, and D) cross-linked binding. Figure 1 presents examples of each of these four cases. A further complexity involves the definition of multiple binding sites per antigen molecule on the cell surface to which the antibody binds. In the discussions that follow, binding sites are considered instead of antigen molecules for simplicity. This complexity was well summarized by Davis et al., 1998, in which it was reported that the number of antibodies that can bind to a specific marker on a lymphocyte, such as CD3, has been reported to vary from 52,000 to 177,000 and for CD4 it can vary from 46,000 to 202,000. Further, it was experimentally demonstrated that the binding of one specific antibody clone, Leu 3a, is bivalent, namely one antibody binds to two distinct CD4 markers; while a second clone, L120 predominately bound in a monovalent manner to the CD4 markers. Finally,
when a recombinant form of the CD4 markers coupled latex bead was used, the bivalent nature of the Leu 3a clone disappeared and the Leu 3a and L120 binding was very similar, which is very likely the direct result of antigen density and membrane fluidity. These observations, combined with other variabilities, led Davis et al. to comment that while the quantitation of the number of antibody epitopes on a cell appears to be a straightforward concept, in practice it is complex and dependent on the specific antigen, antibody, and instrument and that proper controls and understanding are needed.

With respect to the four scenarios presented in Figure 1, in the following discussion we will only consider scenarios A and B. The disassociation constant for scenario A is given by:

\[
K_{D1} = \frac{[Ag][Ab]}{[Ag \cdot Ab]} \tag{5}
\]

and for scenario B:

\[
K_{D1} = \frac{[Ag][Ab]}{[Ag \cdot Ab]}
\]

\[
K_{D2} = \frac{[Ag][Ag \cdot Ab]}{[Ag \cdot Ab \cdot Ag]} \tag{6}
\]

A distinction should be made that while \([Ag]\), and \([Ab]\) represent the global concentration of antigens and antibodies, on the contrary, \([Ag']\) represents the local concentration of antigen binding sites, which is the function of antigen density and membrane fluidity. As reported by Davis et al., for low antigen density cells or fixed
cells, bivalent binding is less likely to happen. Also, bivalent binding is unlikely if $K_{D2}$ is sufficiently high.

A mass balance can be written for scenario A for both the antibody and the antigen:

$$[Ag]_{Total} = [Ag] + [Ag - Ab]$$  \hspace{1cm} (7)

$$[Ab]_{Total} = [Ab] + [Ag - Ab]$$  \hspace{1cm} (8)

and for scenario B:

$$[Ag]_{Total} = [Ag] + [Ag \cdot Ab] + 2[Ag \cdot Ab \cdot Ag]$$  \hspace{1cm} (9)

$$[Ab]_{Total} = [Ab] + [Ag \cdot Ab] + [Ag \cdot Ab \cdot Ag]$$  \hspace{1cm} (10)

When one uses either a flow cytometer, FCM, or cell tracking velocimetry (CTV), to quantify the fluorescence labeling or the magnetophoretic mobility of a cell labeled with a antibody-fluorochrome conjugate, or a antibody magnetic nanoparticle conjugate, respectively, one is not able to distinguish between $[Ag \cdot Ab]$ or $[Ag \cdot Ab \cdot Ag]$. Consequently, the term $[complex]$ is introduced:

$$[complex] = [Ag \cdot Ab] + [Ag \cdot Ab \cdot Ag]$$  \hspace{1cm} (11)

Next, the concept of valance, $\alpha$, is introduced:

$$\alpha = \frac{[Ag \cdot Ab] + 2[Ag \cdot Ab \cdot Ag]}{[Ag \cdot Ab] + [Ag \cdot Ab \cdot Ag]} = \frac{[Ag \cdot Ab] + 2[Ag \cdot Ab \cdot Ag]}{[complex]}$$  \hspace{1cm} (12)

Inspection of Equation 12 indicates that if all the antibodies bind to the cell in a monovalent nature (Scenario A), the concentration of $[Ag \cdot Ab \cdot Ag]$ is zero and the
valance is 1. In contrast, if all the binding is of the homogeneous, bivalent nature (Scenario B), then \([Ag \cdot Ab]\) is zero and the valance is 2. In reality, especially with antibody magnetic colloid conjugates, the actually situation is probably somewhere between 1 and 2.

Substituting Equation (12) into (8):

\[
[Ag]_{total} = [Ag] + \alpha([Ag \cdot Ab] + [Ag \cdot Ab \cdot Ag])
\]  

(13)

and from Equations (5) and (6) one can obtain:

\[
[Ag \cdot Ab] + [Ag_2 \cdot Ab] = \frac{[Ag][Ab]}{K_{D1}} \cdot (1 + \frac{[Ag]}{K_{D2}})
\]  

(14)

If we make the assumption that \([Ag] \ll K_{D2}\), and substitute Equation 13 into 14, after some rearrangement, it can be shown that:

\[
[Ag \cdot Ab] + [Ag_2 \cdot Ab] = \frac{[Ag]_{total} \cdot [Ab]}{K_{D1} + \alpha[Ab]}
\]  

(15)

The assumption normally holds true unless very small \(K_{D2}\) value exists, which represents a highly cooperative bivalent binding. If divided by cell concentration, equation (15) now becomes:

\[
\frac{[\hat{complex}]}{[cell]} = \frac{[Ag]_{total} \cdot [Ab]}{K_{D1} + \alpha[Ab]} = \frac{[\hat{Ag}]_{total} \cdot [Ab]}{\alpha \cdot [Ab]}
\]  

(16)
where \([\hat{\text{complex}}]\) and \([\hat{\text{Ag}}]\) are the number of complexes formed and total antigens per cell. Finally, when conducting an experiment and a saturating amount of antibody is used, one would expect:

\[
\alpha = \frac{[\text{Ag}]_{\text{Total}}}{[\text{complex}]_{\text{max}}} = \frac{[\hat{\text{Ag}}]}{[\hat{\text{complex}}]_{\text{max}}} \quad (17)
\]

Substituting equation (17) into equation (16) for \([\hat{\text{Ag}}]_{\text{Total}}\), a simpler form of equation (16) is obtained:

\[
\theta = \frac{[\text{complex}]}{[\text{complex}]_{\text{max}}} = \frac{[\hat{\text{complex}}]}{[\hat{\text{complex}}]_{\text{max}}} = \frac{[Ab]}{\frac{K_D}{\alpha} + [Ab]} \quad (18)
\]

where \(\theta\) is a measure of the degree of saturation of the antigen sites on the cell surface. Experimentally, \(\theta\) is simply the normalized fluorescence intensity in flow cytometry and normalized mobility in CTV.

**Measurement of receptor-ligand interactions** Flow cytometry (FCM) has been used for determination of binding affinity between cellular receptors and fluorescently labeled ligands [Benedict, 1997]. The amount of ligand receptor complexes formed per cell can be quantified using the measured fluorescence intensity (FI) of the cell. According to Schwartz et al. 1996, the FCM’s response to fluorescence signal (FI) can be described by:

\[
FI = a \cdot \log_{10} (Ab \cdot Ag) + F_{Ig} \quad (19)
\]
if logarithmic amplification is used (typical of instrument produced in the past), or:

\[ FI = a \cdot [Ab \bullet Ag] + Fl_{bg} \]  

(20)

if linear amplification (a practical choice on most new instruments sold currently) is used. In these relationships, \( FI \) is the fluorescence intensity of a labeled cell at certain wavelength, which is unitless, and \( a \) is the conversion factor between fluorescence intensity and the amount of antibody-antigen complexes formed per cell \([Ab \cdot Ag]\). \( a \) is a function of a number of variables including the instrument setting, such as the photomultiplier voltage, as well as intrinsic characteristics of the fluorescent dyes. \( Fl_{bg} \) is the fluorescence intensity of unlabeled cells which is often referred to as cellular autofluorescence. Autofluorescence is a strong function of the excitation wavelength, the detection wavelength, the type of cell, as well and the source of the cells. For example, alveolar macrophages, cells which are obtained from inside human lungs, typically have a higher level of autofluorescence than macrophages obtained from human blood, and alveolar macrophages obtained from the lungs of humans that smoke cigarettes have a further, significantly elevated level of autofluorescence [Pankow, 1995]. This autofluorescence is most pronounced in the emission wavelength of two most commonly used fluorescent dyes, fluorescein isothiocyanate (FITC) and phycoerythrin (PE) [Viksman, 1994].

Alternatively to fluorescent labels, the amount of antibody-antigen complexes formed can also be quantified by magnetophoretic mobility measurements if the antibody is conjugated to magnetic nano particles [McCloskey, 2003]. Magnetophoresis is the
motion of particles in a viscous fluid under the influence of a magnetic energy gradient.

Previous work in our laboratories have demonstrated that a relationship can be written, and experimentally verified over a specific range of values, for the magnetophoretic mobility, \( m \), of an immunomagnetically labeled cell or microbead [McCloskey, 2003]:

\[
m = \frac{ABC \, n_2 \, \Delta \chi V_{\text{nano}}}{3\pi D_c \eta} = \frac{ABC \, n_2 \, \phi_{\text{nano}}}{3\pi D_c \eta} = \beta ABC \phi_{\text{nano}} \tag{21}
\]

\[
ABC = n_1 \theta_1 \lambda_i \tag{22}
\]

\[
\beta = \frac{n_2}{3\pi D_c \eta} \tag{23}
\]

where as before, \( ABC \) is the antibody binding capacity, \( n_2 \) is the number of magnetic nano-particles conjugated to the antibody, \( \phi \) is the field interaction parameter which is the product of the difference in magnetic susceptibility of the nanoparticle and the suspending fluid, \( \Delta \chi \), and the volume of the particle, \( V_{\text{nano}} \). Further, \( D_c \) is the diameter of the cell, and \( \eta \) is the viscosity of the suspending fluid. \( ABC \) can be further defined, as in equation 22, as the product of \( n_1 \), the number of antigen sites per cell, \( \theta_1 \), the fraction of the antigen sites on the cell surface bound (degree of saturation), and \( \lambda_i \), the valence of the antibody. It is noted that \( \lambda = \frac{1}{\alpha} \). The cause of this reciprocal relationship is perspective from which is it defined: \( \lambda_i \) from the perspective of the antigen, \( \forall \) from the perspective of the antibody.
Equation (21) neglects the contribution of the unlabeled cell to the magnetophoretic mobility (the intrinsic magnetophoretic mobility). It is based on the assumption that cells have a very small or negligible intrinsic magnetophoretic mobility. While it is true for most types of cells suspended in buffer (mainly phosphate buffered saline, PBS), it has been reported that the red blood cells show different intrinsic magnetic properties as the result of the oxygenization state of hemoglobin [24]. In addition, Moore et al. 2004 successfully used paramagnetic salts to modify the magnetic property of the suspending buffer to result in higher separation resolution, in which case, the assumption made in Equation (21) is no longer true. To account for the contribution of unlabeled cells to the magnetophoretic mobility, Zhang et al. 2004 modified equation (21) in an analogous manner to the FI relationship:

\[
m = \beta ABC \phi_{nano} + m_0 = b(ABC) + m_0
\]

where

\[
m_0 = \frac{\Delta Z_{cell} \cdot V_{cell}}{3\pi \eta D_c}
\]

Unlike FI measurements, however, measurements of \( m \) are reported in specific units, such as \( \text{mm}^3\cdot\text{T-A/s} \). If all of the terms in \( b \) (Equation 23) and \( m_0 \) are known, the knowledge of the magnitude of \( m \) allows one to determine the value of \( ABC \). This leads to one of the motivations of the current study, namely, the determinations of the binding constants of Equation (1), which governs, along with the concentrations of the respective constituents, the degree of saturation of the antigen sites, \( [Ag]_{\text{total}} \).
Non-ideal antibody binding, the concept of ABC  Ideally, the number of bound antibodies to the cell would equal the number of cell surface antigens. However, as has been presented previously, significant ranges in the number of antibodies that bind to specific surface antigens have been observed and reported. Antibody binding capacity (ABC) has been suggested, and used, as means to begin to take into considerations some of the factors related to this non-ideal relationship (Davis, 1998; Schwartz, 1996). In Equation 22, a relationship was presented which attempts to relates the antibody binding capacity to a number of these non-idealities including: the degree of saturation of antibody binding, $\sigma$ (which is the primary focus of this manuscript), the number of antigen sites, $n_1$, and the valence potential (either $\forall$ or $8$) of the binding. However, in some situations, other factors are involved, including a lack of space, typically referred to as steric hindrance.

Given this background, as written, the $\text{ABC}$ for a given system can very from 0 to a maximum of $\frac{[Ag]_{\text{Total}}}{\alpha}$. To account for this, another variable is introduced, $\varepsilon$, which can vary from $(0 < \varepsilon \leq 1)$ and correspondingly, ABC is now equal to:

$$
\text{ABC} = \theta \cdot \frac{[Ag]_{\text{Total}}}{\alpha} \cdot \varepsilon
$$

(26)

and Equation 16 becomes:

$$
\frac{\hat{\text{complex}}}{\alpha} = \frac{\theta \cdot \frac{[Ag]_{\text{Total}}}{\alpha} \cdot \varepsilon \cdot [Ab]}{K_{D1} \cdot \frac{1}{\alpha} + [Ab]} = \frac{\text{ABC} \cdot [Ab]}{K_{D1} \cdot \frac{1}{\alpha} + [Ab]}
$$

(27)
Under theoretically saturating labeling conditions, $2=1$, and $[Ab] \gg K_{D1}$; therefore Equation (27) becomes:

$$[\text{complex}]_{\text{th,max}} = \frac{1 \cdot \frac{[Ag]_{\text{Total}}}{\alpha} \cdot \varepsilon \cdot [Ab]}{\frac{K_{D1}}{\alpha} + [Ab]} = \frac{[Ag]_{\text{Total}}}{\alpha} \cdot \varepsilon = ABC$$  \hspace{1cm} (28)

Therefore, dividing Equation 27 by 28, one obtains:

$$\frac{[\hat{\text{complex}}]}{[\text{complex}]_{\text{th,max}}} = \theta = \frac{[Ab]}{\frac{K_{D1}}{\alpha} + [Ab]}$$ \hspace{1cm} (29)

In practice, as will be demonstrated experimentally below, it is quite often difficult to predict beforehand whether or not saturation will be reached when an experiment is conducted. If one were to conduct a set of experiments in which the concentration of labeling reagent was varied, one would expect that the ratio of the theoretically maximum value of the complex concentration, $[\hat{\text{complex}}]_{\text{th,max}}$, which is just equal to ABC for that specific combination of antibodies-antigen (Equation 28), to the the experimentally measured maximum concentration of the complex, $[\text{complex}]_{\text{exp,max}}$, would range from 0 to 1. Therefore, the addition of the ratio, $\frac{ABC}{[\hat{\text{complex}}]_{\text{exp,max}}}$, to Equation 29 results in the final form:
\[
\frac{[\text{complex}]^{\text{\hat{}}}}{[\text{complex}]_{\text{exp, max}}} = \theta = \left( \frac{ABC}{\frac{K_{D_{1}}}{\alpha}} \right) \frac{[Ab]}{\frac{[Ab]}{\alpha}} + [Ab]
\]  

(30).

Note, if the experimentally measured complex concentration is equal to the theoretical maximum, then Equation 30 reduces to Equation 29.

4.4 Materials and methods

Preparation of peripheral human blood. Buffy coat prepared from a 500mL blood drawn from a healthy donor was purchased from American Red Cross (Columbus, OH) and diluted with Hanks balanced salt solution (HBSS) (JRH Biosciences, Lenexa, Kansas) at the ratio of 1:2. 22.5mL diluted blood was carefully layered over 17.5mL Accu-Prep lymphocytes Ficoll (1.077 g/mL) density gradient (Accurate Chemical and Scientific Corp., Westbury, NY) in a 50mL conical tube (Fisher Scientific, Pittsburgh, PA), and centrifuged at 400×g and room temperature with brake off for 30 minutes. After centrifugation, the mononuclear cell layer was carefully removed from the interface of plasma and ficoll layer, washed several times by centrifugation at 350×g, 4°C for 5 minutes with a solution consisting of: Dulbecco’s phosphate buffered saline (DPBS modified; JRH Biosciences, Lenexa, Kansas), 2mM ethylene-diamine tetraacetic acid (EDTA; Sigma, St. Louis, MO) and 0.5% fraction V bovine serum albumin (BSA; Sigma, St. Louis, MO). Pellets were then resuspended in labeling buffer, and the cell concentration was determined using a Coulter® multisizer II (Coulter electronics limited, England). The cells were resuspended in medium, RPMI-1640 (ATCC, Manassas, VA), and 10% Fetal bovine serum (FBS; JRH Biosciences, Lenexa, Kansas), and cultured in T-
75 flasks (Fisher Scientific, Pittsburgh, PA) at 37°C, 5% CO₂ to deplete adherent monocytes. After 48 hours’ culture, the medium was carefully removed from the flasks, and Ficoll density separation was performed again to further enrich for the lymphocytes as previously described. After centrifugation, lymphocyte layer was carefully removed and washed in labeling buffer two times. The resulting pellet was then resuspended in labeling buffer and analyzed on the Coulter® multisizerII for the purity and concentration of lymphocytes, which are subsequently referred to as peripheral blood lymphocytes (PBLs).

**Biotinylated microspheres** ProActive® biotin-coated microspheres (catalog number CP10N; Bangs Laboratories, IN, USA) are reportedly uniformly sized polystyrene microspheres, 5.12 µm in diameter, with a standard deviation of 0.1 µm. These microspheres come suspended in 100mM Borate (pH 8.15), 0.1% BSA, 0.05% Tween 20, 10mM EDTA and 0.1% NaN₃.

**Fluorescent labeling of PBLs** Mouse anti-human CD3 antibodies (clone # HIT3a) conjugated to phycoerythrin, PE, and fluorescein isothiocyanate, FITC, from BD Biosciences Phamingen (San Diego, CA) were used to label the PBL. Specifically, the anti-CD3-PE and anti-CD3-FITC antibodies (Cat # 555340, Lot # 0000054407 and Cat # 555339, Lot # M076161) respectively, were reported by the manufacturer to have antibody concentrations of 1.5µg/ml and 50µg/ml, respectively.

PBLs were suspended in PBS at the concentration of 1.0×10⁶ cells per 100 µL. Cells were incubated with appropriate amount of antibodies at 4°C for 15 minutes to ensure that the binding equilibrium has reached. Cells were washed with PBS, by
centrifugation at 350×g, 4°C for 5 minutes. Pellets were then resuspended at the concentration of 1.0×10^6 cells per mL of fixing solution, 2% paraformaldehyde (Mays Chemical, Indianapolis, IN) in PBS.

**Magnetic labeling of Biotinylated microspheres** Two types of commercial, magnetic nano particles bound to streptavidin, SA, were used: MACS™ beads (Catalog number 481-01/02; Miltenyi Biotec, CA, USA) and BD™ Imag SA beads (Catalog number 551308, BD Pharmingen, CA, USA). SA is coated on the outer surface of both nanobeads, which consists of an iron oxide core coated with a polysaccharide, and the MACS bead are reported by the manufacturer to be 50 nanometers in diameter. The BD™ Imag are reported by the manufacturer to be XXX nanometers and by Zhang et al. (2004) to be 231 nanometers in diameter. The SA-MACS™ beads are supplied in a suspension of 0.1% gelatin with 0.05% NaN3, and the BD™ SA nanobeads are suspended in aqueous buffered solution containing 0.09% NaN3, 0.5% BSA and 2mM EDTA.

**Magnetic labeling of PBLs** BD™ Imag mouse anti-human CD3 (clone # HIT3a) nanoparticle conjugates, anti-CD3 – DM, were used to label PBLs. PBLs were suspended in PBS at the concentration of 1.0×10^6 cells per 100 µL. Cells were incubated with appropriate amount of antibodies at 4°C for 30 minutes to ensure that the equilibrium was reached. Cells were then washed with PBS, by centrifugation at 350×g, 4°C for 5 minutes. Supernatants were carefully taken without disturbing pellets for further analysis. Pellets were then resuspended at the concentration of 0.5×10^6 cells per mL of labeling
buffer to ensure that no significant cell-cell interaction is involved [Zhang, 2004]. The measurement of magnetophoretic mobility was performed on CTV.

Fluorescence Intensity measurements The measurement of fluorescence intensity was performed on a FACSCalibur flow cytometry (Beckton Dickinson, Florida, USA), and the data was analyzed using WinMDI version 2.8. This FCM uses logarithmic amplification as described by Equation 19.

Magnetophoretic mobility measurements Magnetophoretic mobility measurements were made on an instrument referred to as a Cell Tracking Velocimetry, CTV, discussed previously [Chalmers, 1999; Zhang et al. 2004]. Briefly, CTV measures the magnetically induced movement of cells or particles, on a cell-by-cell basis, in a well-defined magnetic field. As stated previously, the value of $B$ varies from 1.38 to 1.15 T in the viewing region. However, the gradient of $B$ varies in an opposite manner such that the magnetic energy gradient, $S_m$ (149 T·A/mm²), remains nearly constant in the area where the measurement is made (a rectangular viewing area of 1.66mm×1.23mm). Movement of cells or particles in this area is captured and recorded using an inverted microscope and a 30Hz Cohu CCD 4915 camera (Cohu Electronics, San Diego, CA). The images are digitized into a two dimensional matrix of 640 by 480 pixels by µTech imaging grabbling board (µTech Corp., Billerica, MA). The CTV algorithm, modified from the Particle Tracking Velocimetry, PTV, algorithm, uses five consecutive frames to generate most probable paths of cells or particles. The displacements, more accurately, the traveling pixels of cells or particles in a certain time range are the direct measurement from CTV. Magnetophoretic mobility is obtained with the careful calibration of
dimensions and magnetic field. As a result of continuing improvements, this system is currently semi-automated and an analysis of over 1,000 cells or particles can be performed within 20 minutes.

**Detection of free antibody concentration in suspension** As reported above, in commercial anti human CD3 magnetic particles samples, such as BD™ Imag anti-human CD3 particles (anti-CD3-DM), another protein, usually gelatin or BSA is added in the suspension to stabilize the antibody coated on the particle surface. In an attempt to measure the actual concentration of the antibody nanoparticle conjugate, or SA-nanoparticle conjugate, in equilibrium with the conjugate bound, gel filtration was performed on the equilibrium supernatant before a total protein assay was performed. Two types of gel filtration columns were used: PD-10 column (Amersham Biosciences, NJ, USA) for the smaller, SA-MACS™ particles and a Sepharose™ CL-4B column for the relatively larger Imag particles.

The actual operation of the gel column consisted of introducing 700 µL PBS buffer to the CL-4B or PD-10 column, next 350 µL of the sample to be analyzed, and then 5,950 µL of PBS was added. A total of 20, sequential, 350 µL eluant samples were subsequently collected. Each eluant was then analyzed for protein concentration using Micro BCA total protein assay (Pierce Biotechnology, IL, USA) and Spectra MAX 250 (Molecular Devices, USA) in duplex following manufacturer’s recommendation.

**Curve fitting of data** Two approaches were used to analyze the binding data to determining the binding constants: traditional double reciprocal plots and the use of the Single Rectangular 2 Parameter Hyperbola model:
with the SigmaPlot 8.0 software (SPSS Inc.) This best fit algorithm assumes each data point is equally weighted unlike the double reciprocal plot, which places disproportional weight on the low concentration data points.

4.5 Results

Enrichment of lymphocytes from mononuclear layer obtained after Ficoll density separation To obtain accurate measurements of the binding constants on specific receptors on lymphocytes, it is highly desirable to have a pure cell suspension. While the buffy coat purchased from Red Cross are enriched leukocytes, there is still significant contamination from erythrocytes and monocytes. To further enrich for lymphocytes, Accu-prep, Ficoll density gradient separations as well as 48 hour monocyte depletion cultures were performed on buffy coats.

Figures 2 and 3 present FCM and coulter counter analysis of a typical blood sample at the various stages of enrichment, respectively, and Table 2 quantifies the results. Figure 2 present the progression in the increase in purity can be observed, from Ficoll after obtaining from Red Cross, 2A, 24 hours of culture, 2B, 48 hours of culture, 2C, and after a final Ficoll separation, 2D. A similar progression in purity, from initial Ficoll after obtaining from Red Cross (red line), 24 hours of culture (green line), and 48 hours of culture (blue line). Quantitatively, as presented in Table 2, FCM and coulter counter analysis presents similar increases in purity from 13-16 percent to 78- 80% during this process.
Histograms of lymphocytes labeled with FITC, PE and magnetic nanoparticles. Figures 4A and 4B presents histograms of the FI of purified lymphocytes labeled with anti-CD3-FITC and anti-CD3-PE, conjugated antibodies at concentrations of 1.1µg/mL, 1.2µg/mL, respectively. Figure 4C and 4D are a linear and log histogram presentation of the magnetophoretic mobility of purified, lymphocytes labeled with anti-CD3-DM conjugates at a concentration of 24µg/mL. As will be shown below, these concentration are saturating concentrations. Note the similarity of the percent positive of the three methods of labeling (4A-4C).

Determination of Equilibrium antibody-label conjugate concentrations

Figure 5A is a calibration curve for the MicroBCA protein assay in the form of absorbance at 562 nm as a function of concentration of the provided protein standards. A representative elution curve of anti CD3 DM sample separated on a CL-4B column is presented in Figure 5B. Each solid data point corresponds to an eluant of 350 µL and is plotted in the form of the absorbance of the eluant at 562 nm as a function of total volume eluted from the column. Two peaks are visible, a small peak which corresponds to antibody-nanobead conjugate at approximately 4500-5500 µL which corresponds to the anti CD3 DM beads and a much larger peak at 7900 µL which corresponds to the BSA, which is added by the manufacture to stabilize the suspension. This larger, BSA peak was confirmed by the addition of only BSA (open symbols) to the column in a separate separation experiment. As further confirmation that the antibody-nanobead conjugates elute at 4500-5500 µL, a yellow band, corresponding to the magnetic particles, can be
observed moving through the column at this position. This yellow tint has been observed with a number of different magnetic nanoparticle colloids.

The actual protein concentration of the eluants was determined from the calibration curve presented in Figure 5A. The total protein eluting in a specific peak was approximated using the linear trapezoidal rule. To test the recovery of proteins after eluting from Sepharaose™ CL-4B column, 50µL of albumin standard (2000µg/mL) was eluted through the gel column. After elution and analysis, a recovery of 99% was obtained.

**Development of saturation curves for affinity interactions.** As presented previously, a Langmuir type, saturation relationship is expected when antibody-fluorocrome conjugate, antibody-magnetic nanoparticles conjugate, or SA-magnetic-nanoparticle conjugates are bound to the appropriate cell receptor or surface marker. The assumption of such a relationship is supported both theoretically (Equation 1-2) and experimentally (Comella et al. 2001; Chosy et al. 2003). However, Equation 2 also indicates that the concentration of the antibody, or ligand, is the equilibrium concentration, not the initial concentration. Since a low number of cells were used relative to the total volume of suspending buffer, it was assumed that the initial and equilibrium concentration of the antibody-fluorescent conjugate was the same. (A discussion of this assumption will be made in the discussion section). However, for the studies using antibody or SA magnetic nanobead conjugates, equilibrium concentrations of the labeling reagent were made using the gel filtration and protein assay described above was used.
Since the flow cytometry instrument used in this study uses logarithmic amplification circuits, for each set of labeling studies, the fluorescence intensity, FI, for each data point was normalized by dividing by the highest FI in that set of experiments. Such a normalization procedure results in a relationship similar to Equation 29 and allows experiments on different days to be compared. Figures 6A and 6B contain data from two different studies of the binding of anti-CD3-FITC to purified, human lymphocytes; 6A is a plot of normalized FI as function of equilibrium antibody concentration and 6B is a classic double reciprocal plot of the same data. The two different types of symbols corresponds to two different sets of experiments and the equilibrium antibody concentration was determined using the gel filtration experiments described previously. Figures 6C and 6D, contain data from three sets of experiments, similar to 6A and 6B, except that anti-CD3-PE antibodies were used.

Figures 6E and 6F present a saturation curve and double reciprocal plot of the magnetophoretic mobility of lymphocytes labeled with anti-CD3-Imag conjugates. However, unlike FI measurements, an absolute value of magnetophoretic mobility is obtained from the CTV instrument. For the three sets of experiments performed, two of which obtained saturation values of mobility; 2.35e-04, and 2.38e-04 mm³/T-A-s. Since these maximum values are very similar, a single value of 2.4 x 10⁻⁴ was used to normalized all three sets of experiments.

**Binding of SA-MACS and SA- Imag to Biotinylated microspheres.** Figures 7A and 7B and 7C and 7D present the normalized mobility of the biotinylated
microspheres labeled with different amount of SA-MACS™ beads and SA-Imag beads. Analysis was done similar to what was done for CD3 binding.

4.6 Discussions

Model fitting of the binding data  It can be observed from Figures 7 and 8, equation 29 reasonably fits the data.

When using equation (29) to fit the data, $a$ represents $\frac{ABC}{[complex]_{\text{max}}}$, while $b$ represents $\frac{K_D}{\alpha}$ . It is clearly shown from the figure that the binding curve for anti CD3 PE conjugates hasn’t reached the saturation curve, thus a large $a$ value is obtained. While in the other cases, a smaller $a$ value is obtained. The calculation of antibody binding capacity $ABC$ should be made with the consideration of actual extent of saturation. The fitted $b$ value for anti CD3 FITC conjugate is almost two times lower than that of anti CD3 PE conjugate, and is about ten times lower than that of anti CD3 DM conjugate. To make a comparison of the actual binding dissociation constant, an estimate of binding valence is needed. Since the anti CD3 antibody used in this study is a whole antibody, the valence for FITC and PE conjugates can be approximated as two. Because of the much larger sized DM beads, the valence for DM conjugates can be approximated as one. With the assumptions on valence made, the actual ratio of binding dissociation constants is FITC: PE: DM=1: 2: 5. Converting the unit of binding dissociation constants to moles/liter (MW of antibody is 150000), it is found that they (FITC, $4.67\times10^{-9}$M; PE, $9.34\times10^{-9}$M; DM, $2.67\times10^{-8}$M) all fall into the range listed in Table 1. From the comparison made above, the antibody antigen interaction becomes weaker as the
conjugate size increases. This effect is more clearly shown when streptavidin magnetic bead conjugates are labeled onto biotinylated polystyrene beads shown in figure 8. It is suggested that the steric hindrance effect not only affect the antibody binding capacity as will be discussed as following but also affect the strength of the interaction.

**Antibody binding capacity of the PBL for antiCD3**  
To attempt to quantify the antibody binding capacity, ABC, of the PBL for CD3, two of the three experimental sets presented in Figure 7 were conducted using BD QuantiBrite Calibration beads. Figure 7A presents the FCM histograms of the four beads, each with a different level of PE conjugated to the bead, and Figure 7B presents the linear relationship between mean FI as a function of the PE molecules bound per bead. With this calibration curve, and the assumption that there is one PE molecule bound per antibody, the maximum amount of PE molecules found in experiments was estimated to be 31600 and 30500. Clearly from the data fitting above, the real saturation point is not reached in the experiments, and to account for the difference, the ABC is determined to be 50000 (31600 times a). Anti CD3 FITC antibody binding was not calibrated independently, and the ABC for this antibody is assumed to be the same as that of anti CD3 PE because their very similar size. The maximum amount of anti CD3 DM beads was determined to be 1500, with the property of magnetic nano particle predetermined [Zhang, 2004]. Taking account of the difference between ABC and the maximum amount of beads detected, the ABC for anti CD3 DM beads was calculated to be 1650.

**Scale-up problems in immunological labeling**  
The good agreement between experimental data and Equation (27) suggests that the binding between surface antigens
and antibody conjugates can be described by (28). This not only provides a way to determine the binding affinity in the interaction, it also suggests that a scale up model can be developed to better predict the binding of antibody conjugates to cells of interest. In the effort to deplete T cells (CD3 positive) for bone marrow transplantation, it is typical to handle as much as $10^{10}$ cells. With this high amount of cells to work with, a good estimate of the amount of antibodies needed to achieve the sufficient magnetophoretic mobility should be made, as it is important to achieve high level depletion while make the process economic. Also, a good scale-up model helps our prediction of the cell separation performance in our magnetic cell sorting systems. Here, a scale-up model is proposed, and the estimated value is compared with the experimental data to validate the mathematical model.

From (27),

$$[Ab] = \frac{K_{D1}}{\alpha} \cdot \frac{\theta}{1 - \theta}$$

(30)

$$\therefore [Ab]_{Total} = \frac{\theta}{1 - \theta} \cdot \frac{K_{D1}}{\alpha} + \theta \cdot ABC,$$

(31)

or $$\theta = \frac{([Ab]_{Total} + \frac{K_{D1}}{\alpha} + ABC) - \sqrt{([Ab]_{Total} + \frac{K_{D1}}{\alpha} + ABC)^2 - 4 \times [Ab]_{Total} \times ABC}}{2 \times ABC}$$

(32)

Once the constant $\frac{K_{D1}}{\alpha}$ and the antibody binding capacity $ABC$ are determined from the small-scale experiments, predictions can be made on the total amount of antibody needed to achieve certain fluorescence intensity or magnetophoretic mobility or vise verse based on Equations (31) and (32). To verify the proposed scale-up model
shown above, the same conditions were used to label $1 \times 10^6$, $10 \times 10^6$, and $20 \times 10^6$ similarly purified lymphocytes with the same anti CD3 antibodies we used in the previous study. The measured $\theta$ is compared with what was predicted value using Equation (31) and results from the study, as shown in Figure 9. A close agreement is found, which validates the scale-up model.

Following, we can make a simulation on the amount of antibodies needed to achieve 90% saturation of T cell labeling when working with $10^{10}$ cells. Clearly seen from Figure 10, it is important to suspend cells at high enough concentration in order to decrease the volume of labeling solution, which results in much lower usage of antibodies.

4.7 Conclusions

In this manuscript, a methodology is shown to study the binding of magnetic nano particle conjugated antibody to cellular antigens using CTV. Compared to using fluorescently conjugated antibody, a decrease in binding affinity is found, which can be explained by the sterric hindrance. The most significant effect of using magnetic nano particles conjugated antibody is that the maximum number of antibody antigen complexes formable ($ABC$) decreases dramatically, which is the direct result of relatively large particle size and relatively rigid property of particles. The valance of the binding can be determined by comparing the $ABC$ values for whole antibody conjugate and Fab fragment conjugates [Davis, 1998]. It is obvious that for the purpose of determining the antigen expression level, Fab fragment conjugates should be chosen.
the case of immunomagnetic binding, the valence of binding can be assumed as 1, resulted from the strong sterric hindrance effect.

Using peripheral T cells as an example, we experimentally determined the binding constants and $ABC$, and with these measured parameters we are able to predict the outcome of large scale labeling.

It should be realized though that when small scale binding study is performed to determine the parameters for large scale labeling, caution should be taken so that the amount of antibody conjugates in the system is much higher than the amount needed to reach saturated binding, so that the saturation point determined is only related to the property of cells and antibody conjugates, not the relative amount of antibody conjugates.

4.7 Acknowledgement

This work has been supported by the National Science Foundation (BES-9731059 to J.J.C. and CTS-0125657 to P.S.W.) and the National Cancer Institute (R01 CA62349 to M.Z. and R33 CA81662-01 and R01 CA097391 to J.J.C.).
4.8 References


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Table 4.1 Dissociation constants of some common biological affinity interactions

<table>
<thead>
<tr>
<th>RECEPTOR-LIGAND PAIR</th>
<th>$K_d$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody-antigen</td>
<td>$10^{-7}$-$10^{-11}$</td>
</tr>
<tr>
<td>DNA-protein</td>
<td>$10^{-8}$-$10^{-9}$</td>
</tr>
<tr>
<td>Cell receptor-ligand</td>
<td>$10^{-9}$-$10^{-12}$</td>
</tr>
<tr>
<td>Enzyme-substrate</td>
<td>$10^{-2}$-$10^{-5}$</td>
</tr>
<tr>
<td>Avidin-biotin</td>
<td>$\sim10^{-15}$</td>
</tr>
<tr>
<td>Streptavidin-biotin</td>
<td>$\sim10^{-15}$</td>
</tr>
<tr>
<td>Lectin-monosaccharide</td>
<td>$10^{-2}$-$10^{-5}$</td>
</tr>
<tr>
<td>Lectin-oligosaccharide</td>
<td>$10^{-5}$-$10^{-7}$</td>
</tr>
</tbody>
</table>

Table 4.2 Percentage of lymphocytes in peripheral blood cells after culture and Ficoll separation determined by Coulter counter and flow cytometry.

<table>
<thead>
<tr>
<th></th>
<th>Percentage of lymphocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Coulter Counter</td>
</tr>
<tr>
<td>After Ficoll</td>
<td>15.8</td>
</tr>
<tr>
<td>24 hours culture</td>
<td>18.7</td>
</tr>
<tr>
<td>48 hour culture</td>
<td>26.1</td>
</tr>
<tr>
<td>48 hour culture &amp; Ficoll</td>
<td>77.8</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 4.1 Potential antibody binding scernios on cells.

Figure 4.2 Dot plots of forward light scattering and side light scattering for peripheral blood lymphocytes obtained from Ficoll density gradient (A), after 24 hour culture (B), after 48 hour culture (C), and Ficoll density gradient after 48 hour culture (D).

Figure 4.3 Size distribution of peripheral blood lymphocytes directly after Ficoll density gradient (in red), after 24 hour culture (in green), and after 48 hour culture (in blue).

Figure 4.4 Histograms of peripheral blood lymphocytes with mouse anti human CD3 antibody conjugated to FITC (4A), PE (4B), magnetic nano particles in linear scale (4C) and magnetic nano particles in logarithmic scale (4D). In histograms (4A-4C), two peaks are identified as CD3 negative and CD3 positive. In Figure 4D, only one peak is present, which shows that CD3 negative cells have negative mobility.

Figure 4.5 Calibration curve generated by using the MicroBCA protein assay, which is linear in the range of 0.5~200ug/mL (5A). Elution curves of anti CD3 DM conjugates and standard protein BSA (Mw=66342) (5B). The elution was conducted on a CL-4B Sepharose™ column and the elutes were analyzed using MicroBCA protein assay; each data point corresponds to a 350 µL elution sample. The peak at the elution volume of 4500 ~ 5500 µL corresponds to the elution peak of SA-MACS nanoparticles, which is clearly separated from the peak of BSA, which peaks at around 7900 µL.

Figure 4.6 Saturation curve and double reciprocal plot for peripheral blood labeled with BD™ anti human CD3-FITC, 6A and 6B, BD™ anti human CD3-PE, 6C and 6D, and BD™ Imag anti human CD3 beads-DM, 6E and 6F.

Figure 4.7 FCM histograms, 7A, and calibration curve of QuantiBrite PE Beads, 7B.

Figure 4.8 Saturation curve and double reciprocal plot for biotinylated polystyrene (5.12 micron) beads labeled with streptavidin-MACS nanobeads, 8A and 8B, and streptavidin-DM nanobeads, 8C and 8D.

Figure 4.9 Comparison between predicted $\theta$ and measured $\theta$ when immunological labeling is scaled up.

Figure 4.10 The effect of cell concentration on the amount of antibodies used when $10^{10}$ cells are labeled.
Figure 4.1  Potential antibody binding scenarios on cells.
Continued on page 113
Figure 4.2    Dot plots of forward light scattering and side light scattering for peripheral blood lymphocytes obtained from Ficoll density gradient (A), after 24 hour culture (B), after 48 hour culture (C), and Ficoll density gradient after 48 hour culture (D).
Figure 4.3 Size distribution of peripheral blood lymphocytes directly after Ficoll density gradient (in red), after 24 hour culture (in green), and after 48 hour culture (in blue).
Continued on page 116
Figure 4.4  Histograms of peripheral blood lymphocytes with mouse anti human CD3 antibody conjugated to FITC (4A), PE (4B), magnetic nano particles in linear scale (4C) and magnetic nano particles in logarithmic scale (4D). In histograms (4A-4C), two peaks are identified as CD3 negative and CD3 positive. In Figure 4D, only one peak is present, which shows that CD3 negative cells have negative mobility.
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[anti-CD3-FITC] (µg/mL)

Normalized F/FITC

0.0 0.2 0.4 0.6 0.8 1.0 1.2

6A

\[ a = 1.3 \]

\[ b = 0.35 \mu g/mL \]

[anti-CD3-FITC]^{-1}(mL/µg)

1/normalized F/FITC

0 2 4 6 8 10

6B

Intercept = 0.87
Slope = 0.21\mu g/mL

Continued at page 119
Continued from page 118

**6C**

![Graph showing normalized fluorescence intensity (FIPE) vs. [anti-CD3-PE] (µg/mL)]

- $a = 1.7$
- $b = 0.70 \mu g/mL$

**6D**

![Graph showing 1/normalized FIPE vs. [anti-CD3-PE]$^{-1}$ (mL/µg)]

- Intercept = 0.63
- Slope = 0.38 µg/mL

Continued at page 120

119
Figure 4.6 Saturation curve and double reciprocal plot for peripheral blood labeled with BD™ anti human CD3-FITC, 6A and 6B, BD™ anti human CD3-PE, 6C and 6D, and BD™ Imag anti human CD3 beads-DM, 6E and 6F.
Figure 4.7 FCM histograms, 7A, and calibration curve of QuantiBrite PE Beads, 7B.
[streptavidin] (µg/mL)  

Normalized mobility

$\text{Intercept} = 0.84$  
$\text{Slope} = 7.0$ µg/mL

Continued at page 123
Figure 4.8 Saturation curve and double reciprocal plot for biotinylated polystyrene (5.12 micron) beads labeled with streptavidin-MACS nanobeads, 8A and 8B, and streptavidin-DM nanobeads, 8C and 8D.
Figure 4.9 Comparison between predicted $\theta$ and measured $\theta$ when immunological labeling is scaled up.
Figure 4.10  The effect of cell concentration on the amount of antibodies used when $10^{10}$ cells are labeled.
CHAPTER 5: COMPARISON BETWEEN FLOW CYTOMETRY (FCM) AND CELL TRACKING VELOCIMETRY (CTV) ON QUANTITATIVE CELLULAR RECEPTOR STUDY

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5.1 Abstract

Cellular receptor expression is an important parameter for understanding cell-cell interaction, and disease progression. Flow cytometry has long been used for cellular receptor study. With the use of nano-sized magnetic labels, immunomagnetic cytometry, such as cell tracking velocimetry (CTV) has the potential to be developed as an alternative analytical tool for receptor study. This manuscript focuses on comparing flow cytometry and CTV by using peripheral blood monocytes under the stimulation of lipopolysaccharide (LPS).

The results clearly showed the good agreement in receptor analysis between these two analytical tools. Some differences have also been found between the two. All these observations show that CTV is best suited to analyze cells with high background (autofluorescence) and/or low receptor expression level. Restricted from high autofluorescence, alveolar macrophages meet difficulty in quantifying receptor expression using flow cytometry. CTV was then successfully used to study their basal receptor expression and receptor expression kinetics under the stimulation of LPS.
5.2 Introduction

With the development of science and technology, the study of human diseases and their treatments has more and more been based on the knowledges at the molecular level [Hodge, 2003]. Cells, of course, are complex units, which are far from complete understanding. Meanwhile, studies of some of the “key” players become possible, including some cellular receptors, which are important in cell-cell communications [Desjardin, 2002].

Immunofluorescence technique, such as flow cytometry (FCM), has long been widely used in cell biology study, by quantitating cellular parameters, such as cell size, cell granularity, DNA amount, and receptor expression level [Pankow, 1995]. With a biological sample, one has to face a heterogeneous population containing different cell types, not to mention that cells live different “lives” even in one cell type. FCM measures cellular parameters of multiple cells on a single cell basis, which means a high-resolution picture of cells studied can be obtained, instead of an averaged one. FCM uses fluorescently labeled antibodies to impart cells the ability to “fluorescent” under the excitation of lasers. The ability of cells to “fluorescent” specifically is related to the cellular receptor under study, the antibodies used, and the conjugated fluorescent tag. And this ability is quantified as fluorescence intensity (FI), which is a relative value, and calibration needs to be performed together with the analysis to relate the corresponding MESF (molecules of equivalent soluble fluorochrome) or ABC (antibody binding capacity) to the measured FI value [Schwartz, 1996].
At the same time, paramagnetic labels have been broadly used in applications in biology and medicine [Bjornland, 2003; Ji, 1996; Lea, 1988], which impart labeled cells the ability to magnetophoresis (the motion of an electrically neutral body in a viscous medium when exposed to a magnetic energy gradient) in the same direction as the magnetic energy gradient. Self-developed Cell Tracking Velocimetry (CTV) [Chalmers, 1999] is capable of accurately measuring the magnetophoretic mobility of cells (cell’s induced velocity in a well-defined magnetic field divided by the magnetic energy gradient). The diagram of CTV is shown in Figure 1. Briefly, cells suspended in a fluid, are pumped through a glass tubing into the magnetic field. The movement of cells in the viewing region (1.66mm×1.23mm) is magnified by the inverted microscope, videotaped by a 30Hz Cohu CCD 4915 camera (San Diego, CA), and digitalized into a two dimensional matrix of 640 by 480 pixels by µTech imaging grabbing board (MuTech Corp., Billerica, MA). This corresponds to each pixel representing a “real world” of 2.59 by 2.57 microns. Digitized images are analyzed by CTV algorithm, modified from Particle Tracking Velocimetry, PTV, algorithm [Guezennec, 1994], to locate cells in each frame. Velocities of cells are calculated based on their displacements in certain time period, and converted to magnetophoretic mobility by dividing by the magnetic energy gradient calibrated, for easier comparison between different magnetic fields. CTV measures mobility of cells (unlabeled or labeled) in a well-defined magnetic field on a single cell basis.

Cell’s magnetism after labeling is imparted through the use of antibody magnetic label conjugates. Currently available magnetic labels differ in size and composition.
Based on the difference in size, these labels can be classified as particulate, colloidal and molecular sized labels. Colloidal sized labels are chosen in our application because the relative large ratio in size between cells and labels ensures the linearity between mobility and the cellular receptor expression level in a larger range [McCloskey, 2000] and the relative high magnetic response ensures the significant signal versus noise ratio. The acclaimed diameter of MACSTM (Magnetic Activated Cell Sorting) microbead (Miltenyi Biotec, Auburn, CA, USA) is 50 nm, which is compatible to the size of some important lymphocyte antigenic determinants: a rod of $35 \times 8$ nm for CD45, a rod of $13 \times 3$ nm for CD4 [Moore, 1998].

Magnetophoretic mobility of a labeled cell can be given in expression (1) [Zhang, 2004].

$$m = b \cdot [\text{complex}] + m_{bg}$$

(1)

where $m$ is the magnetophoretic mobility of a labeled cell, $b$ is the linear conversion factor between mobility and amount of magnetically conjugated antibodies formed $[\text{complex}]$ (number of complexes per cell). When it reaches binding saturation, $[\text{complex}]$ equals to $ABC$, the antibody binding capacity. The magnitude of $b$ relates to the labeling strategies used [McCloskey, 2001], magnetic labels used and also the suspending fluid [Zhang, 2004]. $m_{bg}$ is the mobility of unlabeled cells, and in the following context, will be denoted as $m_{unlabeled}$. $m_{unlabeled}$ is the property of certain cell type and the fluid where cells are suspended. In the cell types studied, most of the cells are diamagnetic with respect to the normally used suspending fluid, which is a labeling buffer, Dulbecco’s
phosphate buffered saline (DPBS modified; JRH Biosciences, Lenexa, Kansas), 2mM ethylene-diamine tetraacetic acid (EDTA; Sigma, St. Louis, MO) and 0.5% fraction V bovine serum albumin (BSA; Sigma, St. Louis, MO). As the result, $m_{\text{unlabeled}}$ is a small negative value. One of the exemptions is the red blood cells, which can be either diamagnetic or paramagnetic, depending on the oxygenization state of their hemoglobins [Zborowski, 2003].

With the similarity between immunomagnetic technique and immunofluorescent technique, CTV has the potential to be an alternative tool for FCM on cellular receptor analysis. In this paper, the comparison between FCM and CTV was made with the analysis of receptor expression of monocytes under the activation of lipopolysaccharide (LPS) from *E. coli*.

Monocytes circulate in the blood and act as barriers upon the invasion, especially from bacteria. Compared to other blood cell types, monocytes, cells that have not reached their final states, are very easy to be stimulated [Goldsby, 2000]. Endotoxins present in the medium and the process of their enrichment from whole blood should be strictly controlled for the prevention of their stimulation. For serum used in culture, it should be heat-inactivated to limit the potential function of endotoxins. It has also been found that attachment of monocytes to vessel walls can induce the differentiation, and as the result, nonadherent polypropylene tubes are used in the study. Until now, it is still hard to culture monocytes in vitro without the addition of growth factors, which may prolong the lifespan of monocytes by initiating their differentiation. Besides that, their fate is highly regulated and relates to the invasion of foreign pathogens [Maus, 2001]. CD14 receptor, a
characteristic receptor of monocytes, has long been believed as an important player in the inflammatory response [Yang, 1995; Hopkins, 1995], however, it is also hard to explain how the signal is transmitted since CD14 receptor does not have cytoplasmic tail. The answer becomes clear with the discovery of Toll-like receptors. By now, ten Toll-like receptors have been identified. Toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) are considered as pattern recognition receptors, which contribute in the recognition of invading microorganisms [Oshikawa, 2003; Fan, 2002]. TLR4 is specific to gram-negative bacteria, such as E. coli, while TLR2 is specific to gram-positive bacteria. The activation mechanism is proposed that CD14 receptor recognizes bacteria, and forms a complex. The formation of this complex makes bacteria accessible to toll-like receptors. These Toll-like receptors bind to the specific portion of bacteria, and transmit the signals through their cytoplasmic tails.

In this paper, FCM and CTV were used to study the basal expression of CD14 and TLR4 of monocytes and their expression kinetics when activated using LPS. The results were compared and discussed.

CTV was then used to study receptor expression of human primary alveolar macrophages (AMs), which are known for having high autofluorescence [Pankow, 1995] and have met difficulties in flow cytometry analysis. Attempts to accomplish this problem in FCM include quenching intracellular autofluorescence using crystal violet [Hellden, 1991], using fluorescence dye with higher emission wavelength [Viksman, 1994], and applying cell-cell compensation in flow cytometry data analysis [Lohmeyer, 1994]. As stated earlier, most cell types have a negative intrinsic magnetophoretic
mobility, or automagnetism. This fact leads to the idea of using immunomagnetic method to quantify cellular receptors, which are lowly expressed or/and expressed on highly autofluorescent cells.

5.3 Methods and Materials

**Purification of Monocytes from Peripheral Blood**

Buffy coat was purchased from Red Cross (Columbus, OH). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque density separation. Briefly, blood was mixed with Hanks balanced salt solution (HBSS) (JRH Biosciences, Lenexa, Kansas) at the ratio of 1:2. 22.5mL blood solution was carefully layered on 17.5mL Accu-Prep™ (Accurate Chemical & Scientific Corporation, Westbury, NY) in 50mL polypropylene conical tube (Fisher Scientific, Pittsburgh, PA). Centrifugation was performed at 400×g at room temperature for 30 minutes with break off. The formed PBMC layer was carefully removed and washed twice in labeling buffer and counted with a Coulter® Multisizer II (Counter Electronics, FL). Cells were enriched for monocytes by MACS™ Monocyte Isolation Kit (Miltenyi Biotec, Germany, Cat # 130-091-153), a negative selection kit, according to manufacture’s recommendation. Enriched monocytes were then counted and resuspended for culture.

**Culture of Monocytes together with LPS**

Purified monocytes were resuspended in RPMI-1640 medium (ATCC, Manassas, VA) with 10% heat-inactivated fetal bovine serum (FBS; JRH Biosciences, Lenexa, Kansas) at the concentration of 3×10^6 per mL. 1ng/mL LPS (from *E. coli* strain 0127:B8, Difco, Detroit, MI) was added
to the cell suspension and cells were incubated in polypropylene culture tubes at 37°C and 5% CO₂.

**Analysis of Monocytes Receptor Expression**  At certain time point, cultured monocytes were centrifuged at 37°C, 400×g, for 6 minutes and the supernatant was discarded to rid of LPS. Monocytes were then resuspended in buffer, and labeled with CD14 (FITC conjugated, clone MφP9, Beckton Dickinson, CA) and TLR4 (PE conjugated, clone HTA125, eBioscience, San Diego, CA) for 30 minutes on ice. Flow cytometry analysis was carried out on FACSCalibur flow cytometry (Beckton Dickinson, Florida, USA), and the data was analyzed using WinMDI version 2.8. For CTV analysis, monocytes were further labeled with anti-PE MACSTM microbeads (Miltenyi Biotec, Germany) and/or anti-FITC MACSTM microbeads (Miltenyi Biotec, Germany). Cells were resuspended at 0.5×10⁶ per mL and then subject to CTV analysis.

**Bronchoalveolar Lavage** Bronchoalveolar lavage (BAL) has been used extensively for disease diagnostics [Huaringa, 2000]. BAL was performed in nonsmokers who appear to be healthy at the time of bronchoscopy. Briefly, BAL was carried out via a fiberoptic bronchoscope. The bronchoscope was wedged in one subsegmental bronchus. 0.9% sterile saline solution was instilled and recovered by gentle aspiration. Typically, 30~50mL of BAL fluid was taken depending on the response of donor to the procedure. The fluid was kept on ice before being transferred to authors’ lab for following experiments.
Preparation of Alveolar Macrophages  

Cells were obtained by centrifuging the BAL sample, and resuspended in buffer. Total cell count was performed using Coulter counter. A trypan blue exclusion test for cell viability (>90% in each case) was also performed with the use of hemacytometer. Usually 80% of cells found in BAL sample are macrophages, and there is no need for further purification.

Culture of Macrophages together with LPS  

Macrophages were resuspended in RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS) at the concentration of $0.5 \times 10^6$ per mL. 100ng/mL LPS was added to the cell suspension and cells were incubated in polypropylene culture tubes at 37°C and 5% CO$_2$. Each tube contains $0.2 \times 10^6$ cells.

Analysis of Macrophage Receptor Expression  

As described previously, cells were resuspended in buffer. After immunological labeling step, cells are subject to flow cytometry and CTV analysis accordingly.

5.4 Results

Enrichment of Monocytes by MACSTM Monocytes Isolation Kit  

Figure 2A shows that the CD14 positive cells consists about 18% of PBMCs after Ficoll density separation. Figure 2B shows that after negative magnetic selection, about 87% of PBMCs are CD14 positive. Both two plots are gated for only mononuclear cells (including lymphocytes, monocytes, and a less amount of granulocytes). As further shown from flow
cytometry data on all events in Figure 3A and 3B, the lymphocytes population has been mostly depleted, which is also clearly shown from Coulter counter results in Figure 4.

**Comparison of “autofluorescence” and “automagnetism”**

Under the same instrument settings, fluorescence intensities for different channels of unlabeled monocytes and alveolar macrophages were compared, as shown in Figure 5. Clearly, alveolar macrophages have higher autofluorescence level compared to monocytes in the FL1 and FL2 channel, which is usually used for FITC and PE, two mostly used fluorescent dyes. It is also shown that in FL3, alveolar macrophages don’t show higher autofluorescence than monocytes. Shown in Figure 6, magnetophoretic mobilities of unlabeled monocytes and macrophages almost overlap, which shows that as opposed to flow cytometry, macrophages have similarly low level of automagnetism compared to monocytes.

**Study of the influence of endocytosis on magnetic labeling of cells**

As magnetic particles used in our applications are nano sized, there are concerns that phagocytotic cells, such as macrophages and monocytes, would endocyte these nano particles, which will result in nonspecific binding. Magnetophoretic mobility of unlabeled cells was compared with that of cells incubated together with plain magnetic nano particles (they don’t have affinity to cell surface markers) at 4°C. T test shows that there are almost no difference between these two mobility distributions for monocytes and macrophages. It supports that endocytosis is a process highly dependent on temperature as part of endocytosis process is performed by cell membrane whose fluidity is highly
temperature dependent [Tomoda, 1989]. In the experiments involving immunomagnetic labeling, temperature should be well controlled to ensure that endocytosis is inhibited.

**Comparison between FCM and CTV on determination of percentage of CD14 expression monocytes**

From Figure 8A, the percentage of CD14 positive cells in peripheral blood mononuclear cells determined by CTV is 54%. Figure 8B is different from Figure 2B in that the gating was based on the forward scattering, which correlates with cell size. In CTV, the algorithm used for cell tracking recognizes cells based mainly on the shape and the size, so the comparison between FCM and CTV was made on the same criteria. In Figure 8B, 57% of PBMCs are CD14 positive. The percentage is very close to what was obtained in CTV. The comparison shows that CTV is capable of accurately differentiating positive population and negative population.

**Comparison between FCM and CTV on their sensitivity**

The sensitivities of FCM and CTV were compared by analyzing CD14 (Figure 9) and TLR4 (Figure 10) expression after the incubation with LPS. First of all, in both figures, the results from FCM and CTV are in good agreement. However, in Figure 10A, distinct increase in TLR4 expression is observed after incubation with LPS; and in Figure 10B, the difference is not that obvious.

Figure 9 and 10 show that CTV has a comparable sensitivity as flow cytometry. The difference in receptor expression shown in flow cytometry also can be observed in CTV.

**Comparison between FCM and CTV on quantification of cellular receptors**

Flow cytometry uses fluorescence intensity (FI), a relative value, to express the
magnitude of cellular receptors expressed per cell, which can be used as a measure of cell’s biological state. Calibration is needed every time when such analysis is carried out as there is a variation between machine to machine, operator to operator, and day to day. Microspheres coated with certain amount of fluorochromes (QuantiBrite™ PE beads from Beckton Dickinson, CA were used here) are used to establish the standard curve between the fluorescence intensity (FI) value and the number of fluorochromes [Davis, 1998]. And thus the value obtained is not the true antigen amount, but the antibody binding capacity $ABC$ or the amount of fluorochromes bound per cell. In order to derive the real antigen amount, the binding valance should be evaluated either theoretically or experimentally [Oshikawa, 2003]. On the contrary, magnetophoretic mobility, which relates directly to cellular receptor expression level as found out previously, is an absolute value with the unit of mm$^3$/T-A-s or mm$^3$-s/kg. With the knowledge of magnetic label’s property, we are able to derive how many magnetic labels are bound onto cell surface with no need of calibration. Similarly, valency of binding should also be evaluated in order to know the amount of antigens expressed. In both cases, antibodies should be used at the amount sufficient to reach the saturation state.

Assuming that the binding valency is 1, we calculated the number of TLR4 on monocytes with different culture conditions both in flow cytometry and CTV as shown in Figure 10. The results show a good agreement between these two methods.

**Application of CTV in analyzing alveolar macrophages (AMs)**

Basal expression of CD14, TLR4, and mannose on alveolar macrophages was shown in Figure 11. Mannose receptors are the mostly expressed among the three, followed by TLR4.
During the maturation from monocytes to macrophages, clearly CD14 receptor expression was downregulated, which is in agreement with literatures.

When macrophages were cultured together with 100ng/mL LPS, upregulation of TLR4 was observed first (2 hours typically), followed by the downregulation, as shown in Figure 12A. Mannose receptors gradually down-regulate in the time range of analysis (6 hour), as shown in Figure 12B. CD14 also shows an upregulation at first, but the downregulation happens later than TLR4 (typically 4 hours), as shown in Figure 12C.

5.5 Discussions & Conclusions

**Enrichment of Monocytes by MACS™ Monocytes Isolation Kit**

As can be found out in the flow cytometry data (Figure 3A and 3B), a significant population of granulocytes still exists before and after the enrichment, which shows that the monocytes isolation kit does not work on granulocytes. The isolation kit contains biotin-conjugated anti CD3 (specific for T cells), CD7 (specific for T and NK cells), CD16 (specific for neutrophil and NK cells), CD19 (specific for B cells), CD56 (specific for NK cells), CD123 (specific for basophils) and glycophorin A (specific for red blood cells). The experimental result shows that the composition of isolation kit still has to be optimized for higher monocytes purity. It has been reported that a higher purity of monocytes can be obtained if the positive selection method is used. In this study, negative selection is chosen instead as the result of concerns that binding of magnetic nano particles to cells can activate cells following the culture step. Granulocytes are the most abundant leukocytes in human blood, and theoretically, they should remain at the bottom of tube together with erythrocytes after Ficoll-Paque separation. The flow cytometry result shows
that significant amount of granulocytes are still remained in PBMCs, which can be caused by several possibilities. It could be that the conditions for Ficoll-Paque separation was not best controlled, such as too low temperature and too short centrifugation time. It may also be that during the attempt of isolating PBMC layer, Ficoll layer was mistakenly taken at the same time, which contains granulocytes. However with the same procedure, a lower percentage of granulocytes was obtained when fresh blood was used, compared to buffy coat, which suggests that storage of blood overnight may cause physiological changes of cells.

**Comparison of “autofluorescence” and “automagnetism”**

Cells in most organisms exhibit a natural fluorescence, commonly called “autofluorescence”, from a range of species including metabolites and structural components [Billinton, 2001]. Autofluorescence is cell related property. It has been found that for alveolar macrophages when excited with 488nm argon laser, the most common laser used in flow cytometry, a strong autofluorescence occurs with a peak emission at 541nm and a shoulder at 580nm. The autofluorescence pattern overlaps with that generated by FITC (480 to 580nm), and PE (540 to 640nm) [Hellden, 1991]. The level of autofluorescence is found to be source related too, with the higher autofluorescence level of AM cells found in smokers compared to nonsmokers [Pankow, 1995]. The presence of autofluorescence leads to low signal-to-noise ratio, restriction of sensitivity, and in some cases even failure to differentiate the positively labeled cells from the unlabeled control.

Diamagnetic water accounts for about 70% of weight in a cell [Albert, 1994]. Other important components, such as carbon skeleton, are diamagnetic. For eukaryotic
cells, it has been found that deoxyhemoglobin and methemoglobin are paramagnetic resulting from the unpaired electrons in four heme groups [Pauling, 1936]. As the result, most cells are diamagnetic, except hemoglobin abundant red blood cells at certain oxygenation state. Cells are generally slightly more diamagnetic than buffer, so when suspended in buffer, they tend to stay or move slowly in the direction opposing the magnetic energy gradient, with a near zero magnetophoretic mobility, which can often be neglected. With the use of paramagnetic labels, this leads to a high signal-to-noise ratio.

**Comparison between FCM and CTV on their sensitivity**

The fluorescence intensity (FI) of labeled cells can be expressed in (2) [Schwartz, 1996].

\[ FI = a \cdot ABC + F_{bg} \]  

(2)

where \( FI \) is the fluorescence intensity of a labeled cell at certain wavelength, \( a \) is the linear conversion factor between fluorescence intensity and the antibody binding capacity \( (ABC) \), which is the amount of antibody conjugates bound per cell. \( F_{bg} \) is the fluorescence intensity of unlabeled cells, which is often referred to as cellular autofluorescence.

The sensitivity of FCM can be defined by the smallest amount of fluorochromes bound to the cell that results in a detectable shift in fluorescence intensity (FI) value. Taking the brightest PE as an example, under typical operational conditions, the FI value at different amount of PE molecules for QuantiBrite PE particles is listed in Table 1. And the amount of PE molecule, which results in the shift of one unit in FI value is calculated to be 150.
The sensitivity of CTV is defined by the smallest amount of magnetic labels bound onto the cell surface that could result in a detectable shift. The minimum detectable velocity \( u_{\text{min}} \) can be calculated as in (3) [Nakamura, 2001].

\[
    u_{\text{min}} = \frac{L_{\text{pw}}}{(n_c - 1)t_f}
\]  

(3)

where \( L_{\text{pw}} \) is the distance which represents one pixel in the digitalized image, and it equals to 2.59 microns in current optical system. \( n_c \) equals to 5, because the tracking algorithm uses five consecutive images to get the velocity of tracked particles. \( t_f \) is the time interval between frames. It can be changed by videotaping at different skipping frame number, and ranges from 1/30 second to above for the current 30Hz camera we use. If assuming \( t_f = 1s \), the minimum detectable velocity is calculated to be \( 6.48 \times 10^{-7} \) m/s, which can be normalized to the minimum detectable magnetophoretic mobility of \( 4.5 \times 10^{-6} \text{mm}^3/\text{T-A-s} \). For a cell of 8.5 microns in diameter, if we use the magnetic labels studied [Zhang, 2004], the minimum detectable number of these labels is calculated to be 108.

From the above calculation, the sensitivity of flow cytometry is comparable with that of CTV if PE and magnetic labels studied in [Zhang, 2004] are used. However, PE molecule is by far the brightest chromosome, which means that it has the highest sensitivity of all the available chromosomes. By choosing some labeling strategy to amplify secondary antibody binding [McCloskey, 2001], and using magnetic carriers with larger bead-field interaction constant, the sensitivity of CTV still can be improved.
Figure 9 and 10 show that CTV has a comparable sensitivity as flow cytometry. The difference in receptor expression shown in flow cytometry also can be observed in CTV.

**Comparison between FCM and CTV on quantification of cellular receptors**

The amount of antibody conjugates bound onto the cell (ABC) directly relates to cell’s antigen expression level. It has been found that steric hindrance inhibits the binding of larger magnetic antibody conjugates to cells when high level of antigens is expressed. It follows that at extremely high antigen expression level, use of larger magnetic nano particles, such as the 230nm BD DM beads, can underestimate the amount of antigens expressed. If under this condition, smaller nano particles should be considered, and it also helps to use the two step labeling, instead of one step labeling.

For the purpose of antigen quantification, it works easier if Fab is used instead of the whole antibody, as valency of antibody binding to the antigens can be influenced by many factors, such as antigen density and membrane fluidity.

In conclusion, CTV is demonstrated in this manuscript to have the ability to differentiate positive cells from other cells via the use of magnetic nano particles. The percentage and amount of antigens of positive cells determined by CTV and FCM are in a good agreement. It shows that CTV can be used as an alternative tool to analyze cells with high autofluorescence, such as alveolar macrophages. It also needs to be pointed out that restricted by the relatively larger size of magnetic nano particles compared to fluorescence dyes, CTV is applicable to quantify receptors not highly expressed.
5.6 Acknowledgement

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5.7 Reference


Desjardin, L. E., et al., Mycobacterium tuberculosis-infected human macrophages exhibit enhanced cellular adhesion with increased expression of LFA-1 and ICAM-1 and reduced expression and/or function of complement receptors, Fc γ RII and the mannose receptor, Microbiology, 2002, 148, 3161-71.


<table>
<thead>
<tr>
<th># of PE molecules</th>
<th>Fluorescence Intensity (FI)</th>
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<tbody>
<tr>
<td>863</td>
<td>2.9</td>
</tr>
<tr>
<td>8612</td>
<td>53.8</td>
</tr>
<tr>
<td>31779</td>
<td>197.4</td>
</tr>
<tr>
<td>66408</td>
<td>457.8</td>
</tr>
</tbody>
</table>

Table 5.1 FI values of QuantiBrite™ beads coated with different PE molecule number.
Figure Legends

Figure 5.1 Diagram of Cell Tracking Velocimetry (CTV).

Figure 5.2 CD14 expression results from flow cytometry for peripheral blood mononuclear cells (PBMCs) before (2A) and after (2B) using MACSTM Monocyte Isolation Kit.

Figure 5.3 Side scattering and forward scattering dot plots from flow cytometry of peripheral blood mononuclear cells (PBMCs) before (3A) and after (3B) using MACSTM Monocyte Isolation Kit.

Figure 5.4 Size distributions of peripheral blood mononuclear cells (PBMCs) before (green) and after (red) using MACSTM Monocyte Isolation Kit from Coulter counter.

Figure 5.5 Fluorescence intensity (FI) of FL1, FL2 and FL3 channels for unlabeled monocytes (A, B, C) and alveolar macrophages (D, E, F). As shown, macrophages have much higher autofluorescence level as compared to monocytes.

Figure 5.6 Magnetophoretic mobility of unlabeled alveolar macrophages and monocytes.

Figure 5.7 Influence of endocytosis on magnetic labeling of monocytes (A) and alveolar macrophages (B). As shown, when labeling temperature is set to 4°C, neglectable effect of endocytosis was observed.

Figure 5.8 Monocytes enriched from peripheral blood mononuclear cells (PBMCs) were labeled with FITC conjugated anti human CD14 antibody and CD14 expression was measured both in CTV (Labeled with anti-FITC MACSTM beads) (A) and flow cytometry (B). Histogram (B) was generated using the gating for certain size range. A clear distinction of CD14 positive cells (monocytes) from CD14 negative cells was observed in both methods and the percentage of positive is close for two approaches.

Figure 5.9 Histograms of CD14 expression of enriched monocytes cultured for 2 hours with and without LPS incubation of flow cytometry (A) and CTV (B). In both plots, CD14 expression is upregulated with the incubation together with LPS.

Figure 5.10 Histograms of TLR4 expression of enriched monocytes cultured for 4 hours with and without LPS incubation of flow cytometry (A) and CTV (B). In both plots, TLR4 expression is upregulated with the incubation together with LPS. The number of TLR4 on monocytes was calculated to be 7100 (with LPS) and 3700 (without LPS) in CTV, while the number was 6100 (with LPS) and 3100 (without LPS) in flow cytometry.
Figure 5.11  Basal expression of mannose, CD14 and TLR4 on alveolar macrophages. As shown in (A), clear distinction from control population was achieved for different receptors. Shown in (B), error bar is the standard deviation of 5 experiments.

Figure 5.12  Influence of cultured alveolar macrophage expression of TLR4 (A), mannose (B), and CD14 (C) with and without LPS (100ng/mL).
Figure 5.1  Diagram of Cell Tracking Velocimetry (CTV).
Figure 5.2 CD14 expression results from flow cytometry for peripheral blood mononuclear cells (PBMCs) before (2A) and after (2B) using MACS™ Monocyte Isolation Kit.
Figure 5.3A

Before Enrichment

Figure 5.3B

After Enrichment

Figure 5.3 Side scattering and forward scattering dot plots from flow cytometry of peripheral blood mononuclear cells (PBMCs) before (3A) and after (3B) using MACS™ Monocyte Isolation Kit.
Figure 5.4  Size distributions of peripheral blood mononuclear cells (PBMCs) before (green) and after (red) using MACS™ Monocyte Isolation Kit from Coulter counter.
Figure 5.5  Fluorescence intensity (FI) of FL1, FL2 and FL3 channels for unlabeled monocytes (A, B, C) and alveolar macrophages (D, E, F). As shown, macrophages have much higher autofluorescence level as compared to monocytes.
Figure 5.6 Magnetophoretic mobility of unlabeled alveolar macrophages and monocytes.
Figure 5.7 Influence of endocytosis on magnetic labeling of monocytes (A) and alveolar macrophages (B). As shown, when labeling temperature is set to 4°C, negligible effect of endocytosis was observed.
Figure 5.8 Monocytes enriched from peripheral blood mononuclear cells (PBMCs) were labeled with FITC conjugated anti human CD14 antibody and CD14 expression was measured both in CTV (Labeled with anti-FITC MACSTM beads) (A) and flow cytometry (B). Histogram (B) was generated using the gating for certain size range. A clear distinction of CD14 positive cells (monocytes) from CD14 negative cells was observed in both methods and the percentage of positive is close for two approaches.
Figure 5.9  Histograms of CD14 expression of enriched monocytes cultured for 2 hours with and without LPS incubation of flow cytometry (A) and CTV (B). In both plots, CD14 expression is upregulated with the incubation together with LPS.
Figure 10A

Figure 10B

Figure 5.10  Histograms of TLR4 expression of enriched monocytes cultured for 4 hours with and without LPS incubation of flow cytometry (A) and CTV (B). In both plots, TLR4 expression is upregulated with the incubation together with LPS. The number of TLR4 on monocytes was calculated to be 7100 (with LPS) and 3700 (without LPS) in CTV, while the number was 6100 (with LPS) and 3100 (without LPS) in flow cytometry.
Figure 5.11 Basal expression of mannose, CD14 and TLR4 on alveolar macrophages. As shown in (A), clear distinction from control population was achieved for different receptors. Shown in (B), error bar is the standard deviation of 5 experiments.
TLR4 Expression on Alveolar Macrophages

Mannose Receptor Expression on Alveolar Macrophages

Continued at page 163

162
Figure 5.12 Influence of cultured alveolar macrophage expression of TLR4 (A), mannose (B), and CD14 (C) with and without LPS (100ng/mL).
6.1 Abstract

The feasibility of separation proteins via the use of magnetic colloidal and molecular labels was studied. In the study, biotin conjugated protein was first coated onto the microsphere surface, and the number of proteins coated was determined using immunfluorescent technique. CTV was used to measure the resulted magnetophoretic mobility of microspheres labeled with streptavidin DM beads (colloidal label) and avidin ferritin (molecular label) to determine the particle field interaction parameter for labeled proteins.

Results shows that colloidal labels can be used to separate protein via magnetic labeling, while molecular labels used in this study don’t show significant magnetism and protein separation via the use of this kind of labels apparently will not work. Different magnetic label preparation method should be considered in order to use molecular sized labels for protein separation.
6.2 Introduction

With the development of nano technology, devices down to micron size have found many applications [Furdui, 2001; Choi, 2001]. One of the advantages is that these devices offer information from small sample in a short time.

Magnetic separation has been applied for isolating certain cell types from a heterogeneous mixture [Safarik, 1999; Despres, 2000], purification of proteins [Bucak, 2003; Tanyolac, 2001; Tong, 2001], and isolation of DNAs [Sonti, 1997]. The fact that proteins can be magnetically separated in their native state suggests the possibility of developing this technology into conformation dependent immunoassay (CDI), which has become a hot topic since the observation that the disease causing infectious prion (PrP\textsuperscript{SC}) only differs from the normal prion (PrP\textsuperscript{C}) in its secondary structure [Ye, 2003; Safar, 2002; Loredana, 2002].

Currently, protein separations are mostly performed with the use of micron sized magnetic labels. The difficulty of performing the separation at nano scale is caused by the weak magnetic force magnetic nano labels can impart to the protein relative to the Brownian force (or thermal force). Compared to a few years ago, make of a narrow channel for separation is no longer an obstacle now, which means with the narrow channel, we can achieve much higher magnetic energy gradient with the same magnets, which directly results in stronger magnetic force with the same magnetic labels. In this study, proteins are labeled with colloidal and molecular sized magnetic labels, and the resulted magnetism of proteins is experimentally determined. Based on the information, we are able to design the channel and magnets needed to achieve separation, which is
compared to the dimensions current technology can offer to decide the feasibility of building such a system.

The retention parameter, $\lambda$, used in the field-flow fractionation community, is the ratio of thermal energy to the work required to drive the particle or molecule across the channel thickness.

$$\lambda = \frac{kT}{F_w} \quad (1)$$

where $k$ is Boltzmann’s constant ($1.381 \times 10^{-23}$ J/K), $T$ is the absolute temperature (298K for 25°C), $F$ is the force imparted on the particle or molecule through the external field, and $\omega$ is the channel thickness.

When a particle is placed in a magnetic energy gradient, a magnetic force, $F_{mag}$, is induced, which is defined by the following relationship:

$$F_{mag} = \Delta \chi V \left( \frac{B^2}{2\mu_0} \right) \quad (2)$$

where $\Delta \chi$ is the difference in volumetric magnetic susceptibility between the particle, $\chi$, and the suspending fluid, $\chi_f$, $V$ is the volume of the particle, $B$ is the magnetic field induction, and $\mu_0$ is the magnetic permeability of free space.

Resulted from the viscosity of the suspending fluid, the drag force on the particle is given by equation (3).

$$F_d = 3\pi \nu d \eta \quad (3)$$
where \(d\) is the diameter of the particle, \(\eta\) is the viscosity of the suspending fluid, and \(v\) in this case is the induced velocity of the particle in the field.

By balancing \(F_{\text{mag}}\) and \(F_d\), the induced velocity of the particle in the field, \(v\) is given by:

\[
v = \frac{(\chi - \chi_f) \cdot V}{3\pi d \eta} \left| \nabla \left( \frac{B^2}{2\mu_0} \right) \right|
\]

(4)

This induced velocity, \(v\), can be divided by \(S_m\), the magnetic energy gradient, equal to \(\left| \nabla \left( \frac{B^2}{2\mu_0} \right) \right|\), to obtain the magnetophoretic mobility, \(m\), as shown in equation (5):

\[
m = \frac{(\chi - \chi_f) \cdot V}{3\pi d \eta}
\]

(5)

When a particle is coated with proteins, the magnetophoretic mobility can be described as in equation (6).

\[
m = m_0 + \frac{n \cdot (\chi_p - \chi_f) \cdot V_p}{3\pi d \eta} = m_0 + \frac{n \cdot \phi_p}{3\pi d \eta}
\]

(6)

where \(m_0\) is the intrinsic magnetophoretic mobility of the particle, the product of the relative volumetric magnetic susceptibility of protein and the volume of protein is called the particle-field interaction parameter for certain protein \(\phi_p(\equiv \Delta \chi V)\). And \(n\) is the number of proteins coated onto the particle. If the mobilities of uncoated particle and coated particle are known, \(\phi_p\) can be determined via equation (7).

\[
\phi_p = \frac{3\pi (m - m_0) d \eta}{n}
\]

(7)

Based on the derivation shown previously, the magnetic force per protein is:
\[ \overrightarrow{F}_p = \phi_p \cdot S_m \]  \hspace{1cm} (8)

Substitute equation (8) back into (1),

\[ \lambda = \frac{kT}{\phi_p \cdot S_m \cdot w} \]  \hspace{1cm} (9)

For significant retention, \( \lambda \) should be smaller than 0.1. As shown in equation (9), in order to increase the value of \( \lambda \), \( \phi_p \) and \( S_m \) should be maximized. \( \phi_p \) is the property of protein, and the molecular magnetic labels used. \( S_m \) depends on the magnetic field strength, and also the gap distance, which relates to the channel thickness \( \omega \).

Substituting the definition for \( S_m \),

\[ \lambda = \frac{kT}{\phi_p \cdot \left( \nabla B^2 \right) \cdot w} = \frac{2\mu_0 kT}{\phi_p} \cdot \frac{l}{B^2 \cdot w} \]  \hspace{1cm} (10)

where \( l \) is the gap length for magnetic field induction ranging from \( B \) to 0 T.

With the recent applications of magnetic separation, a variety of magnetic labels can be obtained via commercial sources or research collaborations. In this study Imag DM beads from Beckton Dickinson, CA, and ferritin from Sigma, MO were used to impart magnetism to proteins.

6.3 Material & Methods

**Coating of proteins onto microsphere**

Carboxylate-modified microspheres (Bangs Laboratories, Fishers, IN, Catalog # PC06N/5570) have the mean diameter of 5.25\( \mu \)m (standard deviation 0.64\( \mu \)m). Mouse IgG conjugated with biotin (BD Biosciences Pharmingen, CA, Catalog # 555747) was used to coat the microsphere. Buffer preparation and coating procedure followed the protocol provided by Bangs.
Laboratories. Briefly, the microspheres were first washed in deionized (DI) water several times by centrifugation at 500×g for 8 minutes. The pellet was then washed twice and resuspended in the activation buffer solution (MES buffer, 19.2g/L 2-(N-morpholino) ethanesulfonic acid hemisodium salt (Sigma, St. Louis, MO, Catalog # M8250), adjusted to desired pH range of 4.5~7.5 with 1N HCl or 1N NaOH). To the well suspended solution, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was slowly added to activate the carboxyl terminals on the surface of microspheres and the mixture was allowed to react for 15 minutes at room temperature with continuous mixing. The activated microspheres were then washed twice in coupling buffer (borate buffer, 12.4g/L boric acid (Sigma, Catalog # B-6768), 19.1 g/L sodium tetraborate (Sigma, Catalog # B-9876), adjusted to desired pH range of 7.2~8.5 with 3M NaOH). Mouse IgG biotin conjugates were added to the suspension and allowed to react at room temperature for four hours with constant mixing. The mixture was washed and resuspended in quenching solution (30mM glycine (Matheson Coleman & Bell, Catalog # 2384), 1% fraction V bovine serum albumin (BSA; Sigma, St. Louis, MO)), and mixed gently for 30 minutes. Finally, microspheres were washed and resuspended in storage buffer (Dulbecco’s phosphate buffered saline (DPBS modified; JRH Biosciences, Lenexa, Kansas), 0.5% BSA) at 4°C until used.

**Determination of the amount of proteins coated to the microspheres** Flow cytometry analysis was used to determine if proteins are coated to the microsphere and the extent of the coating. 0, 1, and 2µL Streptavidin-phycoerythrin (PE) (BD Pharmingen CA, Catalog # 554061) were incubated with 10⁵ microspheres suspended in 10µL storage
buffer at 4°C with shaking for 30 minutes. After wash, these labeled microspheres were subjected to flow cytometry analysis on FACSCalibur flow cytometry (Beckton Dickinson, Florida, USA), and the data was analyzed using WinMDI version 2.8. QuantiBrite™ PE calibration beads (Beckton Dickinson, Florida, USA) were used to relate measured fluorescent intensity to PE molecule amount.

**Magnetophoretic mobility measurement of microspheres**

Magnetophoretic mobility measurements were made on an instrument referred to as a Cell Tracking Velocimetry, CTV, discussed previously [Chalmers, 1999; Moore, 2000]. Briefly, CTV measures the magnetically induced movement of cells or particles, on a cell-by-cell basis, in a well-defined magnetic field. As stated previously, the value of $B$ varies from 1.38 to 1.15 T in the tracking region. However, the gradient of $B$ varies in an opposite manner such that the magnetic energy gradient, $S_m = \frac{\nabla B^2}{2\mu_0}$ (149 T·A/mm²), remains nearly constant in the area where the measurement is made (a rectangular viewing area of 1.66mm×1.23mm). Movement of cells or particles in this area is captured and recorded using an inverted microscope and a 30Hz Cohu CCD 4915 camera (Cohu Electronics, San Diego, CA). The images are digitized into a two dimensional matrix of 640 by 480 pixels by µTech imaging grabbling board (µTech Corp., Billerica, MA). The CTV algorithm, modified from Particle Tracking Velocimetry, PTV, algorithm [Guezennec, 1994], uses five consecutive frames to generate most probable paths of cells or particles. The displacements, more accurately, the traveling pixels of cells or particles in a certain time range are the direct measurement
from CTV. Magnetophoretic mobility is obtained with the careful calibration of dimensions and magnetic field. As a result of continued improvements, this system is semi-automated and an analysis of over 1,000 cells or particles can be performed within 20 minutes.

0, 2, 5, 10 and 50µL streptavidin Imag™ beads (Catalog number 551308, BD Pharmingen, CA, USA) were incubated with 10⁶ microspheres suspended in 60µL storage buffer at 4°C with shaking for 30 minutes. Similarly, 0, 1, 5, and 20µL avidin ferritin conjugates (Sigma-Aldrich, Catalog # A4030-IMG) were incubated with 10⁶ microspheres suspended in 60µL storage buffer at 4°C with shaking for 30 minutes. After wash, these labeled microspheres were suspended in buffer at 0.5×10⁶ particles/mL and subjected to CTV analysis.

6.4 Results

Figure 1 shows the size distribution of coated microspheres measured by Coulter counter, and an aggregates peak was observed, which may be explained by too long reaction time for the coupling step. No disaggregation of microspheres was performed as both FCM and CTV algorithms can gate for microspheres of certain size range.

Figure 2A is the histogram of fluorescence intensity for microspheres labeled with different amount of streptavidin-PE. Clearly, with the amount of streptavidin-PE used increasing, the mean fluorescence intensity increases, which can be more clearly seen in Figure 2B. Using the calibration curve generated as in Figure 3A and 3B, we are able to calculate that the mean number of PE molecules per microsphere is 4621. From the communication with the vendor, the ratio of biotin to mouse IgG is 10, and the ratio of
PE to streptavidin is 1. If it is assumed that the one streptavidin binds to one biotin molecule, we can calculate that the mean number of mouse IgG coated on the microsphere is 462.

Figure 4A and 4B is the magnetophoretic mobility of microspheres labeled with different amount of streptavidin DM beads. Clearly, with the amount of streptavidin DM beads used increasing, mobility histogram of microspheres shifts rightward. As can be found from Figure 4B, $m_0$ is $-1.83 \times 10^{-5} \text{mm}^3/\text{T-s}$, and $m_{\text{max}}$ is $2.0 \times 10^{-4} \text{mm}^3/\text{T-s}$. Using equation (7), the particle field interaction parameter for labeled protein $\phi_p$ is calculated to be $2.14 \times 10^{-23} \text{ m}^3$.

Figure 5 shows the magnetophoretic mobility distributions of microspheres labeled with different amount of avidin ferritins, and t test tells that no significant difference can be found for the three distributions.

6.5 Discussion

As discussed earlier, in order to safely say that the separation can be performed, $\lambda$ should be smaller than 0.1. If we substitute the values known and determined when streptavidin DM beads were used back into equation (10), following restriction is obtained.

$$\lambda = 4.82 \times 10^{-4} \cdot \frac{I}{B^2 \cdot w} < 0.1$$

(11)

It thus follows,
\[
\frac{l}{B^2 \cdot w} < 207 \quad (12)
\]

Also, \( \frac{l}{w} > 1 \) (the range of this ratio is determined by the current polymer processing technology)

If the magnetic field strength \( B = 1T \), the requirement shown in expression (12) can be satisfied easily. If the magnetic field strength decreases to \( 0.1T \), the requirement can still be satisfied. So it is obvious that using magnetic nano labels, proteins can be magnetically separated based on how many magnetic labels are bound.

However, magnetophoretic mobility of avidin-ferritin labeled microspheres cannot be differentiated from that of unlabeled microspheres, which suggests that the magnetism of ferritin is too small that CTV is not sensitive enough to detect the minor change in magnetism. Using the data from Zborowski, 1995, the particle field interaction parameter of magnetoferritin is \( 7.8 \times 10^{-28} \text{ m}^3 \). Considering the magnetification factor caused by the ratio of biotin to protein, and the ratio of ferritin to avidin, it is estimated that the labeled protein can have the particle field interaction parameter of \( 1.6 \times 10^{-26} \text{ m}^3 \). It can be found that equation (10) cannot be satisfied if proteins are labeled with ferritin.

From the brief comparison, it is clear that by carefully choosing the field strength, dimension and the magnetic labels, the requirement for having sufficient retention can be achieved, which proves that protein separation can be performed with colloidal size magnetic labels in a sub micron scale separator. Use of ferritin does not provide significant magnetism to achieve sufficient retention, which suggests different label
preparation method should be considered to have a molecular sized labels with higher magnetism.

6.6 Acknowledgement

This work has been supported by the National Science Foundation (BES-9731059 to J.J.C. and CTS-0125657 to P.S.W.) and the National Cancer Institute (R01 CA62349 to M.Z. and R33 CA81662-01 and R01 CA097391 to J.J.C.).
6.7 Reference


**Figure Legends**

| Figure 6.1 | Size distribution of coated microspheres measured from Coulter counter |
| Figure 6.2 | Protein coated microspheres labeled with streptavidin PE conjugates and the FI measured by flow cytometry (2A), and the relation between FI and the amount of streptavidin PE conjugates (2B). |
| Figure 6.3 | Measured FI values for QuantiBrite PE calibration beads at FL2 (3A), and the established calibration curve between FI and the amount of PE molecules (3B). |
| Figure 6.4 | Measured magnetophoretic mobility distributions of coated microspheres labeled with streptavidin DM beads (4A) and the relation between mobility and the amount of streptavidin DM beads (4B). |
| Figure 6.5 | Measured magnetophoretic mobility distributions of coated microspheres labeled with avidin ferritin. |
Figure 6.1  Size distribution of coated microspheres measured from Coulter counter
Figure 6.2 Protein coated microspheres labeled with streptavidin PE conjugates and the FI measured by flow cytometry (2A), and the relation between FI and the amount of streptavidin PE conjugates (2B).
Figure 6.3A

Figure 6.3B

Figure 6.3 Measured FI values for QuantiBrite PE calibration beads at FL2 (3A), and the established calibration curve between FI and the amount of PE molecules (3B).
Figure 6.4 Measured magnetophoretic mobility distributions of coated microspheres labeled with streptavidin DM beads (4A) and the relation between mobility and the amount of streptavidin DM beads (4B).
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<table>
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</table>

Figure 6.5 Measured magnetophoretic mobility distributions of coated microspheres labeled with avidin ferritin.
CHAPTER 7: THE USE OF IMMUNOMAGNETIC METHOD FOR
ENRICHMENT OF BACILLUS CEREUS SPORES

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7.1 Abstract

Dried milk products and infant foods are frequently contaminated with *Bacillus cereus*. The bacteria form spores to escape from the processing procedure, including pasteurization. Pre-enrichment of *Bacillus cereus* is a necessary step in the detection of the bacteria. This study aims to establish a procedure to enrich *Bacillus cereus* spores immunomagnetically for following easier detection.

Two step labeling strategy was used to make *Bacillus cereus* spores magnetic. Mouse anti-*Bacillus cereus* spore antibody, a generous gift from Dr. Charles L. Turnbough Jr., and Dr. John F. Kearney (University of Alabama, Birmingham), together with rat anti-mouse IgG2a+b MACSTM microbeads (Miltenyi Biotec, Germany) were used to impart magnetism to spores. Samples were incubated with antibodies for sufficient time and the following enrichment of spores were performed on a MS separation column (Miltenyi Biotec, Germany). The effluents were analyzed for the presence of *Bacillus cereus* spores by plating on agar plates.

Experimental result shows that by optimizing the condition of incubation of *Bacillus cereus* spores with antibodies, high recovery of spores from the sample was achieved. The resulted enriched sample provides a relatively easy way to detect such spores.

A modified separation process was also tried, and proved to be working, though with lower separation efficiency, which suggests more studies need to be done to have a better understanding of this process.

184
7.2 Introduction

In the food production industry, contamination due to bacteria is a large concern. Harmful organisms can be introduced from daily foods cows consume, soil, dust and air. Therefore, the source of the pathogenic organisms cannot be completely eliminated. One of these bacteria is *Bacillus cereus*, which at large concentrations (>10⁶ per gram) can cause food poisoning with symptoms including watery diarrhea, cramps, and acute nausea [MMWR, 1994]. *Bacillus cereus* is aerobic, spore forming, gram negative bacteria commonly found in meats, milk, vegetables, rice, and etc. It is a member of a genetically closely related group of Bacillus species that include *B. anthracis*, *B. mycoides* and *B. thuringiensis*. Some genetic evidence suggests that these may all be one species [Becker, 2003]. As spores, the bacteria can withstand almost any environment, with the exception of strong acidity. The normal food production procedure, including pasteurization and radiation, does not affect the viability of these spores, which makes it hard to eliminate the potential danger from the food production. *Bacillus cereus* has been recognized as an agent of food poisoning since 1955. Between 1972 and 1986, 52 outbreaks of food-borne disease associated with *B. cereus* were reported to the CDC, but this is thought to represent only 2% of the total cases, which have occurred in that time, and there are some reports on the food poisoning caused by *Bacillus cereus* recently. Given the high standards the Food and Drug Administrations (FDA) has set for food safety, it is necessary that the industry develops technology to test the quality and composition of its products. Taking *Bacillus cereus* as a studying subject also helps our
understanding on *B. anthracis*, which has raised concerns recently because of the potential of being used as biowarfare.

Besides the contamination from the raw material, and production procedure, transportation and storage can either introduce the bacteria or allow it to grow in the ambient environment. However, the detectable concentration currently available methods can provide usually is a lot higher than the concentration limit required for food safety. Immuno-magnetic separation (IMS) has been developed and widely used for many types of separations [Bucak, 2003; Zigeuner, 2003; Despres, 2000]. In food science, IMS has been successfully used for the detection of *Listeria monocytogenes* in cheese [Uyttendaele, 2000]; the detection of *E. coli* O157:H7 from poultry carcass rinse [Lekowska-Kochaniak, 2002]; and the detection of Salmonella [Cudjoe, 1994]. As an easy and fast separation method, IMS is introduced here to first enrich the organisms in the bulk samples for later easy detection. Plating on agar plates was chosen to detect *Bacillus cereus* spores in samples after immunomagnetic separation.

In this manuscript, nano sized magnetic particles were used to impart magnetism to spores as compared to the commonly used micron sized magnetic particles used in other applications [Uyttendaele, 2000; Lekowska-Kochaniak, 2002, Cudjoe, 1994]. MS separation column (Miltenyi Biotec, Germany) was used for enrichment of *Bacillus cereus* spores. However, instead of following manufacturer’s recommendation, the separation procedure was modified to be more economic, and the results were compared with what was obtained following manufacturer’s recommendation.
7.3 Materials & Methods

*Bacillus cereus strains*  
*Bacillus cereus* strains are the gift of Dr. Zeigler (*Bacillus* genetic stock center, the Ohio State University). Four strains (6A1, 6A3, 6A5, and 6A10) were studied.

**Cultivation of Bacillus cereus**  
*Bacillus cereus* strains were cultured in 2×SG medium [Leighton, 1971]. 16.0g Difco nutrient broth (Fisher Scientific, Pittsburgh, PA), 2.0g KCL, 0.5g MgSO$_4$$\cdot$7H$_2$O were added in one liter of deionized water. The medium was adjusted for pH to 7, and autoclaved. After the medium was cooled, pre-filtered 1M Ca(NO$_3$)$_2$, 0.1M MnCl$_2$$\cdot$H$_2$O, 1mM FeSO$_4$, and 50% (w/v) glucose were added at the amount of 1, 1, 1, and 2mL for one liter of medium. *Bacillus cereus* cells were cultured in the medium at 37°C with shaking at 150 rpm.

**Harvest of Bacillus cereus spores**  
*Bacillus cereus* strains were cultured for 48 hours to ensure sporulation, and the glucose concentration was monitored by YSI glucose analyzer (YSI, USA) during the culture. 10mL of medium was removed from the flask, and washed with addition of Dulbecco’s phosphate buffered saline (DPBS modified; JRH Biosciences, Lenexa, Kansas) for 8 times by centrifugation at 500×g for 8 minutes, and the resulted pellet was resuspended in PBS.

**Counting of Bacillus cereus spores**  
*Bacillus cereus* spore suspension was serially diluted with PBS, and 20μL of $10^{-2}$, $10^{-4}$, $10^{-6}$ dilutions were plated in duplex on agar plates. Caution was taken not to spread the inoculums to the edge of agar plates, which may cause difficulty later in colony counting. The plates were kept in an upright
position until the inoculums have been absorbed by the medium. The plates were then inverted and cultured in 37°C incubator overnight. The colonies formed on the plates were counted, and the concentration of spores in the suspension was calculated based on the colony number reading between 20 to 200.

**Labeling of Bacillus cereus spores** 100 and 1000 Bacillus cereus spores per mL suspensions were obtained for each strain based on the spore concentration obtained in the previous step. Mouse anti Bacillus cereus antibody was the generous gift from Dr. Charles L. Turnbough Jr., and Dr. John F. Kearney (University of Alabama, Birmingham). 1g/mL Mouse anti Bacillus cereus antibody was incubated with the spore suspension at 4°C for 30 minutes. 20µL/mL rat anti-mouse IgG2a+b microbeads (Miltenyi Biotec, Germany) were then added into the spore suspensions and the incubation was carried out at 4°C for 30 minutes.

**Separation of Bacillus cereus spores** MS separation column (Miltenyi Biotec, Germany) was used to separate labeled Bacillus cereus spores following manufacture’s recommendation. Before applying the spore suspension to the column, 2mL PBS was used first to wash the column. 1mL spore suspension was carefully added to the column, and the eluted solution was collected as the negative portion. 1mL PBS was then used to flush to column to reduce the nonspecific retention of cells in the column, and the elute was also collected in the negative portion. The column was then removed from the magnets, and 500µL PBS and plunger were used to force the retained cells out, and the elute was collected as positive portion. Two portions were used to plate in duplex to determine the number of spores wherein.
Modified procedure for separation of *Bacillus cereus* spores

The standard procedure for MS separation is to first label the sample together with antibody. In the food industry, it is common that a large volume sample needs to be analyzed. It has been demonstrated that the binding of antibody to the spores is a function of antibody concentration during labeling [Chosy, 2003; Comella, 2001]. What it means is that to effectively label the spores, it is necessary to use large amount of antibody when processing large volume sample in order to increase the chance that antibody reacts with spores (concentration). As what we all aware of is that antibody is expensive, it is desirable to use as little antibody as possible and still have the effectiveness in spore recognition. In the modified procedure, mouse anti *Bacillus cereus* antibody and rat anti-mouse IgG2a+b microbeads were incubated first at 4°C for 30 minutes and the resulted antibody solution was added to the column placed in the magnets and the elute was collected for future use, which may contain the unreacted mouse anti *Bacillus cereus* antibody. The spore suspensions were applied directly to the “loaded” column, and the elute was collected as the negative portion, and when the column is removed from the magnets, plunger was used to get the elute. The elute was centrifuged, and the resulted pellet was resuspended in PBS for spore enumeration by plating method, while the supernatant can be reused to load the column. To study the effect of retention time on the separation, pump was used to control the flow rate. The diagrams of two separation procedures are shown in Figure 1 and 2.
7.4 Results

**Cultivation of Bacillus cereus**  After 48 hour culture, the glucose concentration in the medium is very low, and it is safe to believe that sporulation has occurred in the medium.

**Counting of Bacillus cereus spores**  Bacillus cereus is a motile organism, and the colony formed on the plates shows spreadlike morphology, which means the organism tends to reach out for foods.

**Separation of spores**  The separation results from the standard procedure described above were listed in Table 1. The separation efficiency was calculated by dividing the number of spores found in positive portion with the total number of spores found in two portions after separation. All the strains we studied were shown to have some extent of retention in the column, which means that the antibody we used has sufficient affinity to recognize the spores. However, the existing spores in the negative population indicate that the separation should be further optimized.

The separation results from the modified procedure were listed in Table 2. In addition, the recovery of separation was calculated by dividing the total number of spores found in two portions with the initial count of spores. Different flow rate was used to test for the effect of retention time on the chance of spores to get recognized by the antibody complexes loaded in the column. It can be found from Table 2 that when decreasing the flow rate to increase the retention time of Bacillus cereus spores in the column, the separation efficiency can be increased. Also, in the experiment to change the amounts of
antibodies used, we found that there is no significant change in separation efficiency, suggesting that the amounts of antibodies we used are sufficient.

7.5 Discussions

Even with relatively high amount of antibodies, we haven’t achieved stable and high separation efficiency. It can be because that the antibodies do not have high enough affinity, and most likely it is resulted from the choice of nano particles. Superparamagnetic MACS™ beads don’t have sufficiently high magnetic moment to impart to labeled spores, and thus could not retain all the spores even though they are positively labeled. It is shown that using the standard procedure suggested by the vendor, we achieved higher separation efficiency compared to what we can get from the modified procedure. And changing the amount of antibodies doesn’t have significant effect on separation efficiency. When the modified process was used, the separation column can be viewed as an affinity column, the interactions on spores only dependent on the strength of the antibody antigen interaction. Without further study, it is hard to tell whether the preloading of antibody complexes has effect on the binding affinity of anti Bacillus cereus antibody to the spores.

In conclusion, this study demonstrates that Bacillus cereus spores can be immunomagnetically enriched with the use of nano magnetic particles. To increase the separation efficiency, magnetic nano particles should be carefully chosen in order to impart higher magnetism to spores. The modified separation process proves to be working, but further studies need to be carried out to study the distribution of loaded
antibody complexes in the column and the effect of preloading on antibody-antigen interaction to optimize the process.

7.6 Acknowledgement

Authors want to thank Dr. Charles L. Turnbough Jr., and Dr. John F. Kearney from University of Alabama for providing anti *Bacillus cereus* antibody.
7.7 Reference


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<th>Strain</th>
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<th>(+) Count</th>
<th>(-) Count</th>
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(+) Number of spores remained in the column (magnetic)

(-) Number of spores flown through the column (nonmagnetic)

*, the initial volume of spore suspension subject to separation is 1mL

Table 7.1 Separation results of *Bacillus cereus* spores on MS magnetic column with the procedure recommended by the vendor.
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Table 7.2 Separation results of *Bacillus cereus* spores (strain # 6A3) on MS magnetic column with the procedure modified.
Figure 7.1  The diagram of separation procedure
Figure 7.2 Modified separation procedure
CHAPTER 8: CONCLUSIONS & FUTURE WORK

8.1 Conclusions

In this study, a more sophisticated model has been established to account for the contribution of intrinsic parameters of cells to magnetophoretic mobility of labeled cells, which makes mobility analogous to fluorescence intensity expression well established for immunofluorescently labeled cells. Magnetic susceptibility modified suspending fluid was used to change the mobility of unlabeled spheres and spheres labeled with colloidal labels, so that the magnetic susceptibility of spheres and colloidal labels can be determined. Four commercial colloidal labels were studied. Good agreement of data points with the proposed model was observed for unlabeled polystyrene microspheres, and the determined susceptibility value falls in between the literature values, which validates our model. Particle field interaction parameters for four colloidal labels are estimated together with the independent size measurement done on a dynamic light scattering instrument. The particle field interaction parameter determined for MACS™ beads was comparable with what was determined by other researchers in previous studies. With no other literature values to compare with, results were qualitatively verified when comparing lymphocytes being labeled with two colloidal labels studied. Author used the interaction of biotin and streptavidin to “visualize” colloidal labels in CTV system, and this approach can be applied to other systems, which shows that this
methodology can be further developed in use for routine characterization of magnetic colloidal labels.

The binding model proposed by author to describe antibody binding to surface antigens of cells is verified by data obtained using anti CD3 antibody conjugates (FITC, PE, and DM beads) to label peripheral blood lymphocytes. The obtained parameters show that conjugation of relatively larger DM beads to antibody weakens the antibody antigen interaction and a tremendous deduction on the amount of DM beads can be bound (ABC value for DM beads) is also observed, which is most likely caused by the sterric hindrance. Together with the calibration of ABC value for three conjugates, an estimate is made when immunological labeling was scaled up to 40 million cells, and the measured values have a good agreement with the predicted values, which shows the validity of using the scale up model proposed in future study. This study established a complete and sophisticated way of doing antibody binding study for immunological labeling taking peripheral blood lymphocytes being labeled with anti CD3 antibody as an example.

Considering the similarity between immunofluorescent technique and immunomagnetic technique, researchers have long considering of developing a magnetic cytometry as an alternative to flow cytometry to solve some challenges faced in flow cytometry, such as high autofluorescent cells, i.e. Alveolar Macrophages (AM). Author started the study by comparing automagnetism (cell’s intrinsic magnetophoretic mobility) and autofluorescence, and found that most cells have negligible automagnetism, while cells differ in range and extent of autofluorescence. This observation leads to the
comparison between FCM and CTV on cellular antigen quantification taking peripheral blood monocytes as an example. It was found that CTV and FCM have good agreement on determining the percentage of positive cells and quantifying the number of antigens on cell surface (not highly expressed). The sensitivity of both methods were also discussed and compared, and it shows that both methods have similar sensitivity. CTV was then applied to study AM cells, and was successful in differentiating the positively labeled cells from the unlabeled control. The amount of receptors determined by CTV is in agreement with literature values. CTV was also used to study the receptor expression kinetics of AM cells when LPS was used to mimic the invasion of microbes.

CTV was used to determine how magnetic a single protein can be when it is labeled with colloidal magnetic labels (DM beads in this study) and molecular magnetic labels (ferritin from Sigma in this study). To visualize proteins, proteins (conjugated with biotin) are first coated onto the microsphere surface (-COOH modified surface). The amount of proteins coated was determined by labeling microspheres with streptavidin-PE, and measuring the resulted fluorescence intensity. Calibration was done using QuantiBrite™ PE calibration beads. Magnetophoretic mobility of microspheres labeled with streptavidin DM beads and avidin ferritins were determined independently. Based on the knowledge of how many proteins coated per microsphere, the particle field interaction parameter was determined for labeled proteins, which was then used to calculate the retention factor to see if magnetic force imparted on proteins via labeling can be significantly higher than the thermodynamic force. It shows that proteins labeled with colloidal labels have magnetic force much higher than the thermal force, which
shows using colloidal labels proteins can be separated. However, proteins labeled with molecular labels don’t show significantly higher magnetic force than thermal force, which suggests that proteins cannot be magnetically separated using this approach.

Immunomagnetic cell separation also has been applied to separate *Bacillus cereus* spores from foods with the use of MiniMACSTM column and colloidal magnetic labels. Certain extent of separation was observed. In the modified procedure, MiniMACSTM column was modified as an affinity column to reduce the amount of antibodies needed. Although some extent of separation was found, the separation efficiency is lower than what was found in the original procedure.

### 8.2 Future work

Further development of characterization method for different types of magnetic labels is needed. In chapter 3, author established a methodology on magnetic label characterization. It should be noticed that a higher sensitive CTV system should be developed in order to characterize magnetic labels of interest. It was shown in Chapter 6 that CTV was not capable of detecting magnetophoretic mobility change when molecular labels were used. In the direction of developing a nano scale device for detection, improvement should be made on CTV system.

A multiparameter CTV system should be considered for future development. Author tried to use the addition and extraction method to qualitatively determine the expression of two receptors, and was successful. This approach can be further developed and applied in the future. It is also possible to introduce more parameters in CTV system.
with the use of combination of magnetic labels of different magnetic response pattern. Some theoretical consideration should be made on this potential approach.

In all our studies, antibody or protein conjugated magnetic labels are used. It is possible to use other molecules for molecular recognition, such as DNA. Researchers tried to use DNA to differentiate proteins by their secondary configuration, which makes it a good candidate to be used in conformation dependent immunoassay (CDI).
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206


APPENDIX A: PREPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

Reagents:
1. Fresh blood obtained from volunteer donor by venous puncture or source leukocytes (buffy coat) purchased from American Red Cross
2. Sterile Ficoll-Paque density separation medium (1.077g/mL)
3. Sterile phosphate buffered saline (PBS)
4. Sterile Hank’s Balanced Saline Solution (HBSS)

Procedures:
1. Mix 1:2 blood to HBBS (15 ml blood with 30 ml HBSS) in a 50 ml Falcon tube.
2. Layer 22.5 blood solution over 17.5 ml Ficoll-Paque density separation medium in 50 ml Falcon tube; repeat.
3. Centrifuge at room temperature with brake off at 1350 RPM for 30 minutes.
4. Carefully remove all but 3 –5 ml of the top plasma layer and discard.
5. With separate 10 ml pipette, remove lymphocyte layer taking care to avoid contamination from the red cell layers and add to a 50 ml Falcon tube.
6. Wash cells at least two times in PBS at 1350 RPM, room temperature, for 6 minutes.
7. After the last wash, resuspend pellet carefully in PBS, or medium (RPMI-1640 with 10% FBS) if cells are to be cultured. Cell counting can be done in both Coulter counter and hemacytometer.
APPENDIX B: MICROBCA PROTEIN ASSAY

Reagents:

1. Albumin Standard Ampules, 2 mg/mL, 10×1 mL ampules, containing bovine serum albumin (BSA) at 2.0 mg/mL in a solution of 0.9% saline and 0.05% sodium azide (Catalog # 23209, Pierce Biotechnology, Inc., Rockford, IL, USA)

2. Micro BCA™ Reagent A (MA), containing sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2 N NaOH (Catalog # 23231, Pierce Biotechnology, Inc., Rockford, IL, USA)

3. Micro BCA™ Reagent B (MB), containing 4% bicinchoninic acid in water, (Catalog # 23232, Pierce Biotechnology, Inc., Rockford, IL, USA)

4. Micro BCA™ Reagent C (MC), containing 4% cupric sulfate, pentahydrate in water, (Catalog # 23234, Pierce Biotechnology, Inc., Rockford, IL, USA)

5. Phosphate buffered saline (PBS)

Procedures:

1. Preparation of Diluted Albumin (BSA) Standards

Use Table 1 as a guide to prepare a fresh set of protein standards. Dilute the contents of one Albumin Standard (BSA) ampule into several clean vials, preferably using the same diluent as your sample.

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of Diluent (µL)</th>
<th>Volume of BSA Source (µL)</th>
<th>Final BSA Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>900</td>
<td>100 of stock</td>
<td>200</td>
</tr>
<tr>
<td>B</td>
<td>1000</td>
<td>250 of A</td>
<td>40</td>
</tr>
<tr>
<td>C</td>
<td>500</td>
<td>500 of B</td>
<td>20</td>
</tr>
<tr>
<td>D</td>
<td>500</td>
<td>500 of C</td>
<td>10</td>
</tr>
<tr>
<td>E</td>
<td>500</td>
<td>500 of D</td>
<td>5</td>
</tr>
<tr>
<td>F</td>
<td>500</td>
<td>500 of E</td>
<td>2.5</td>
</tr>
<tr>
<td>G</td>
<td>600</td>
<td>400 of F</td>
<td>1</td>
</tr>
<tr>
<td>H</td>
<td>500</td>
<td>500 of G</td>
<td>0.5</td>
</tr>
<tr>
<td>I</td>
<td>500</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

2. Preparation of the Micro BCA™ Working Reagent (WR)

Use the following formula to determine the total volume of WR required:

\[
\text{total volume WR required} = (\# \text{ standards} + \# \text{ unknowns}) \times (\# \text{ replicates}) \times (\text{volume of WR per sample})
\]
Note: 150 µl of WR is required for each sample in the Microplate experiment. Prepare fresh WR by mixing 25 parts of Micro BCA™ Reagent MA and 24 parts Reagent MB with 1 part of Reagent, MC (25:24:1, Reagent MA:MB:MC). Note: When Reagent MC is initially added to Reagents MA and MB, a turbidity is observed that quickly disappears upon mixing to yield a clear-green WR. Prepare sufficient volume of WR based on the number of samples to be assayed. The WR is stable for several days when stored in a closed container at room temperature (RT).

3. Microplate procedure
A) Pipette 150 µL of each standard or unknown sample replicate into a microplate well.
B) Add 150 µL of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.
C) Cover plate and incubate at 37ºC for 2 hours.
D) Cool plate to RT.
E) Measure the absorbance at or near 562 nm on a plate reader.
F) Subtract the average 562 nm absorbance reading of the Blank standard replicates from the 562 nm reading of all other individual standard and unknown sample replicates.
G) Prepare a standard curve by plotting the average Blank-corrected 562 nm reading for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.
APPENDIX C: COATING PROTEINS TO CARBOXYL-MODIFIED MICROSPHERES

Reagents:
1. Carboxyl-modified microspheres (Diameter 5.25±0.64 µm, P(S/5.5%DVB/5%MAA), Catalog # PC06N/5570, Bangs Laboratories, Inc., USA)
2. Activation buffer (pH 4.5-7.5): MES Buffer, pH range 5.2-7.2 – per 1000 mL pure water, 19.2 g MES free acid (MW 195.2), adjust pH with 1 N HCl or 1 N NaOH.
3. Coupling buffer (pH 7.2-8.5): Borate, pH 8.5 – per 100 mL pure water, 0.62 g boric acid, 0.28 g sodium tetraborate, adjust pH with 1 N HCl or 1 N NaOH.
4. Water soluble carbodiimide (WSC): EDAC, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
5. Protein or other biomolecule with –NH₂ terminus
6. Quenching solution: 30-40 mM glycine, 0.05-1% (w/v) blocking molecule (such as BSA)
7. Storage buffer (pH 7-7.5) with 0.01-0.1% (w/v) blocking molecule

Procedures:
1. Wash 1mL (100 mg/mL) of microspheres 2× in 10 mL of activation buffer.
2. After second wash, resuspend pellet in 10 mL of activation buffer, ensuring that the microspheres are well suspended. (Vortexing, sonication, or rolling should aid in resuspension.)
3. While mixing, add 100.0 mg of WSC* (Addition of WSC may cause clumping; this is generally not a cause for great concern, and should be resolved by incubation with the biomolecule.)
4. Allow to react for 15 minutes at room temperature (18-25ºC), with continuous mixing.
5. Wash 2× in coupling buffer and resuspend in 5 mL of same. As much as possible, ensure that the particles are well suspended, as in Step 2.
6. Dissolve protein (1-10× excess of calculated monolayer**) in 5 mL coupling buffer. Combine microsphere suspension and protein solution.
7. React at room temp. for 2-4 hrs with constant mixing.
8. Wash, resuspend in 10 mL of quenching solution, and mix gently for 30 minutes. Wash, and resuspend in storage buffer to desired storage concentration.
9. Store at 4ºC until used.

*Reaction rate after addition of WSC is pH dependent (as the pH decreases, the reaction rate increases).
**Covalent coupling protocols have historically focused on the binding of a monolayer of protein. The amount of protein that will comprise a monolayer will depend upon factors such as the molecular weight of the protein and its relative affinity for the bead. This amount may be estimated through use of the following equation:

\[ S = \frac{6}{\rho_s d} \cdot C \]

where: \( S \) = amount of representative protein required to achieve surface saturation (mg protein/ g microspheres); \( \rho_s \) = density of solid sphere (g/cm\(^3\)); \( d \) = mean diameter (\( \mu \)m); \( C \) = capacity of microsphere surface for a given; protein (mg protein / m\(^2\) of sphere surface).

For BSA: \( C \sim 3 \) mg/m\(^2\), for bovine IgG: \( C \sim 2.5 \) mg/m\(^2\), and by comparing the MW of your ligand to that of BSA and IgG, surface saturation of other ligands can be approximated.

The actual reaction is shown as below:
APPENDIX D: CTV (CELL TRACKING VELOCIMETRY) STANDARD OPERATION PROCEDURE

1. Setup
1.1 Camera, Microscope and Computer
   a. Verify the cable has been connected between computer and camera.
   b. Use level to verify camera alignment.
   c. Turn on microscope light.
   d. Open Wseq program located on the desktop of computer.
   e. Select the Adjust Video icon (blue icon) to display the current view. The Gain and Offset can be adjusted in this window. Typically set Gain to 2.00 and Offset to 0.03.

1.2 Buffer Bump
   a. Turn valve to open buffer tube.
   b. Turn buffer pump and watch at waste exit to check if there is any leakage or bubbles.
   c. Stop pump and discard waste from waste collection beaker as necessary.

1.3 Sample Loading

This section describes procedures to load the cell sample into the CTV. Refer to the Harvard Syringe Pump Manual for additional details on operation of the pump.

   a. Turn the power of the syringe pump on. Hit any key to stop pump display from flashing.
   b. Verify infuse rate and refill rate. Rates can be viewed or changed by pressing Set followed by Infuse Rate or Refill Rate. The rate is input with the numeric pad. Refer to syringe pump manual for further details. Press Enter after entering desired rate.
   c. Loosen nuts on either side of the slide block and grip knobs on top to pull block back. Remove syringe from pump and discard.
   d. Fill a new syringe with buffer and carefully remove all bubbles from inside of syringe by tapping.
   e. Place a few drops off buffer from the syringe into the valve fitting and replace syringe, expelling any remaining liquid into fitting.
   f. Replace the sliding block and retighten the two nuts to secure syringe.
   g. Turn valve to allow buffer to flow past (not into) syringe.
   h. Turn on the buffer pump and tap tubing/valves to remove any bubbles in the system. Once clear, turn buffer pump off.
   i. Switch valve so that sample will be fed into the syringe.
j. Replace waste collection beaker with the tube, which contains sample. (Note: Be sure to mix sample well before placing in the system.)

k. Select Refill on the syringe pump and press Run/Stop to start the pump. When the reader gets to about 0.2 mL, stop and check images on the computer.

l. If cell concentration is low, keep advancing until more cells are being pulled into the system. If no cells are visible, ‘Infuse’ sample and try one of the following:
   1. Mix sample and reload;
   2. Switch the valve for the buffer the run through, replace the sample tube with waste collection beaker and run the buffer pump to check for bubbles.

m. Once a good density has been found, use the focus knob on the top of the CTV to find the best view.

n. Continue to ‘Refill’ until reader is 0.3 mL.

2.0 Image Grabbing
The Wseq program is used to store images in the RAM and transfer them to the hard drive. Each image uses 300 kB of memory. The RAM is large enough to hold nearly 360 images (12 seconds of images). This will vary depending on the memory usage in the computer. To skip frame with the CTV, record with a skipping frame.

   a. In the Wseq program, select Options, Set Record Options. Enter the number of frames to be recorded and skip frame (i.e. enter 1 to record without a skip frame, 2 to record every other image, etc.). Select OK to return to main window. A memory allocation warning will appear if the amount of RAM required to store the images increases. Select Yes when prompted to turn to the main window.
   b. Select the Adjust Video icon to display the current view. Adjust the Gain and Offset as needed. Verify a good density of cells visible and image is in focus. If needed, pump a fresh sample of cells into viewing area. Select OK to return to main window.
   c. Select the Record Video icon. This opens the Sequence Recorder window. To record, select the triangular Play icon.
   d. After recording, select the Recorded View icon (film clip) to check the image for bubbles, lines, etc. Re-record if image quality is not satisfactory.
   e. If image quality is acceptable, exit sequence recorder and select Save As from the File menu to save images to the hard drive. Save image as D:\CTV\Imag\trial_name set_number.seq. For example, aa01.seq corresponds to trial aa and set 01. The Wseq software allows a maximum of 4 characters in the save name.
   f. Pump a fresh sample into the viewing area.
   g. Repeat steps a-f as needed. Typically, 20-30 sets of images are collected per trial.

3.0 Image Processing
The image processing program contains 4 steps: user setup, image acquisition, image processing.

   a. Open CTV New program located on the desktop.
b. Input the name of data file you want, which does not need to match the name of images recorded.

c. Select the User Setup tab:
   1. If a User File already exists, select Load from User File to open. Verify proper settings.
   2. If a User File does not exist, input 1.72 mm for X and 1.27 mm for Y in the Meas Dimension box.
   3. Enter the Frame Number you want to process (i.e. 1-20)
   4. Input the Particle Parameters in the Data Extraction box. (Parameters are defined in)
   5. Select Use New Values. Important: Just because value changed in User Setup does not mean it has changed everywhere else until Use New Values is selected.
   6. The User File can be saved by selecting Save into User File.

d. Select CDs or HDs to get images from

e. Select To Memory to transfer images to RAM (recommended). Can also work from hard drive by selecting To Disk (not recommended).

f. Select Mutech format.

g. Use browser to select the images to be opened. Only need to select one of the images to open the set. Select Open.

h. The image is now in the RAM and ready for processing.

7. Input the Particle Parameters in the Data Extraction box. (Parameters are defined in)

8. Select Use New Values. Important: Just because value changed in User Setup does not mean it has changed everywhere else until Use New Values is selected.

9. The User File can be saved by selecting Save into User File.

   1. To subtract the background, input the Start, Skip, and End Frames in the Subtract Images box. Select Replace under Save Images to overwrite the original images and save memory. Select Sub Now!.

   2. The images are now ready for data extraction.

i. Select the Delete files, and Merge files, and a data file is generated, which can be used in statistical softwares to generate a histogram of mobilities.