INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®
THEORETICAL AND EXPERIMENTAL CHARACTERIZATION OF MAGNETOPHORETIC MOBILITY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
Kara E. McCloskey, M.S.

The Ohio State University 2001

Dissertation Committee:
Dr. Jeff Chalmers, Adviser
Dr. David Tomasko
Dr. James Rathman
Dr. Kurt Koelling
Dr. Bob Brodkey

Approved by
Adviser
Department of Chemical Engineering
ABSTRACT

Magnetic cell separation technology has become a commonly used technique to enrich or deplete cells of interest from a heterogeneous cell population. One important aspect of continuous magnetic cell separation is the degree to which a cell binds paramagnetic material. It is this paramagnetic material which imparts magnetophoretic mobility to the target cell sufficient enough to allow an effective separation. The methodology and mathematical theory have been developed which address the characterization of magnetic labeling and the fundamental parameters controlling the magnetophoretic mobility of an immunomagnetically labeled cell. Several of the parameters controlling magnetophoretic mobility have been experimentally evaluated using cell tacking velocimetry (CTV) technology and these experimental results were found to be in good agreement with the theory. This characterization of magnetophoretic mobility has provided a further understanding of the paramagnetic labels used in magnetic separation technology and also allows predictions of magnetic cell separation performance.
Dedicated to my parents
ACKNOWLEDGMENTS

The successful completion of this dissertation could not have been accomplished without the understanding, insight, brainstorming, and feedback all of my research group members; Lee Moore, Kristin Comella, Mauricio Hoyos, Steve Williams, Alex Rodriguez, Diane Leigh, Masa Nakamura, Julia Chosy, and Kristie Melnik, and especially my advisers, Dr. Jeff Chalmers and Maciej Zborowski.

The FACS data in this thesis was obtained with the help of Amy Raber, Analytical Cytometry Operator at The Cleveland Clinic Foundation and Andrew Oberyszyn, Analytical Cytometry Operator at The Ohio State University and Diane Leigh.

I would especially like to thank Dr. Abe Schwartz, Flow Cytometry Standards Corporation and Howard Shapiro, whose communications have significantly contributed to my understanding of immuno-labeling techniques and the success of this work. I would also like to thank Dr. George Stark and Dr. Ramana for sharing their transformed fibrosarcoma cell line.

On a more personal level, I would also like to thank my mom, dad, sisters, and others close to me for being a constant source of encouragement and admiration.
VITA

February 14, 1972 ........................................... Born – Washington, Pennsylvania, USA

Summer, 1993 ................................................... Undergraduate Researcher,
The Ohio State University

Summer, 1994 ................................................... Undergraduate Researcher,
The Ohio State University

September, 1994 — March, 1995 ................... Student Engineer,
Technegas, Columbus, Ohio

Summer, 1995 ................................................... Student Engineer,
Ford Motor, Sandusky, Ohio

June, 1996 ......................................................... B.S. Chemical Engineering,
The Ohio State University

March, 1999 ..................................................... M.S. Chemical Engineering,
The Ohio State University

June, 1996 — March, 1999 ......................... Graduate Teaching and Research Associate,
The Ohio State University

April, 1999 — present ........................................ Research Associate,
The Cleveland Clinic Foundation
PUBLICATIONS

Research Publications


FIELDS OF STUDY

Major Field: Chemical Engineering

Specialization: Biomedical Engineering
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>iii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>iv</td>
</tr>
<tr>
<td>Vita</td>
<td>v</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xii</td>
</tr>
<tr>
<td>List of Nomenclature</td>
<td>xviii</td>
</tr>
<tr>
<td>Chapters:</td>
<td></td>
</tr>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2. Magnetophoretic Mobilities Correlate to Antibody Binding Capacities</td>
<td>6</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>6</td>
</tr>
<tr>
<td>2.2 Mathematical Model of Magnetophoresis</td>
<td>8</td>
</tr>
<tr>
<td>2.3 Experimental Methods</td>
<td>15</td>
</tr>
<tr>
<td>2.3.1 Quantum Simply Cellular Microbeads</td>
<td>15</td>
</tr>
<tr>
<td>2.3.2 Primary Labeling Microbeads by Immunofluorescent Antibody</td>
<td>16</td>
</tr>
<tr>
<td>2.3.3 Secondary Labeling Microbeads by Immunomagnetic Antibody</td>
<td>16</td>
</tr>
<tr>
<td>2.3.4 Antibody Saturation Studies</td>
<td>17</td>
</tr>
<tr>
<td>2.3.5 The CTV Apparatus</td>
<td>17</td>
</tr>
<tr>
<td>2.3.6 CTV Analysis</td>
<td>18</td>
</tr>
<tr>
<td>2.4 Results</td>
<td>19</td>
</tr>
<tr>
<td>2.5 Discussion</td>
<td>29</td>
</tr>
<tr>
<td>2.6 Acknowledgements</td>
<td>32</td>
</tr>
</tbody>
</table>
### 3. Measurement of CD2 Expression Levels of IFN-α Treated Fibrosarcomas Using Cell Tracking Velocimetry

3.1 Introduction ................................................................................................. 33
3.2 Mathematical Model of Magnetophoresis .............................................. 36
3.3 Experimental Methods .............................................................................. 39
   3.3.1 Fibrosarcoma Cell Line and Culturing Conditions .................. 39
   3.3.2 Treating Cells with IFN-α ....................................................... 40
   3.3.3 Cell Harvesting .......................................................................... 41
   3.3.4 Cell Size Analysis ...................................................................... 41
   3.3.5 Antibody Saturation Studies ................................................... 41
   3.3.6 Immunofluorescent Labeling the Fibrosarcoma Cells for FCM Analysis .............................................................................. 42
   3.3.7 Immunomagnetic Labeling the Fibrosarcoma Cells for CTV Analysis .............................................................................. 43
   3.3.8 Immunofluorescent Labeling the Fibrosarcoma Cells for CD2 Quantitation using FCM .................................................. 44
   3.3.9 Quantum Simply Cellular Microbeads ...................................... 45
   3.3.10 Immunofluorescent Labeling the Microbeads for CD2 Quantitation using FCM ............................................................ 46
   3.3.11 The CTV Apparatus ................................................................. 46
   3.3.12 CTV Analysis ............................................................................ 47
3.4 Results .......................................................................................................... 48
3.5 Discussion ................................................................................................. 63
3.6 Acknowledgements ................................................................................... 67

### 4. Mobility Measurements of Immunomagnetically Labeled Cells Allow Quantitation of Secondary Antibody Binding Amplification

4.1 Introduction ................................................................................................ 68
4.2 Mathematical Model of Magnetophoresis ............................................. 71
   4.2.1 Methods for Calculation Secondary Antibody Binding Amplification ............................................................ 74
4.3 Materials and Methods ............................................................................. 80
   4.3.1 Cell Preparation .......................................................................... 80
   4.3.2 Scheme 1 ................................................................................... 81
   4.3.3 Scheme 2 ................................................................................... 81
   4.3.4 Scheme 3 ................................................................................... 82
   4.3.5 Scheme 4 ................................................................................... 82
   4.3.6 Antibody Saturation Studies .................................................. 83
   4.3.7 Immunofluorescent Labeling of Quantum Simply Cellular Microbeads ............................................................ 85
   4.3.8 Cell Concentration Effects on Mobility ................................ 86
   4.3.9 The CTV Apparatus ................................................................. 87
   4.3.10 CTV Analysis ............................................................................ 88
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.2.3 Antibody Saturation Studies</td>
<td>158</td>
</tr>
<tr>
<td>7.2.4 Immunofluorescent Labeling the Microbeads</td>
<td>158</td>
</tr>
<tr>
<td>7.2.5 Immunofluorescent Labeling the Cells</td>
<td>159</td>
</tr>
<tr>
<td>7.2.6 Immunomagnetic Labeling QSC Microbeads with CD34+ MACS Isolation Kit</td>
<td>159</td>
</tr>
<tr>
<td>7.2.7 Immunomagnetic Labeling QSC Microbeads with CD34FITC and anti-FITC MACS</td>
<td>160</td>
</tr>
<tr>
<td>7.2.8 Cell Separation</td>
<td>160</td>
</tr>
<tr>
<td>7.3 Results</td>
<td>161</td>
</tr>
<tr>
<td>7.4 Discussion</td>
<td>174</td>
</tr>
<tr>
<td>8. Conclusions and Future Work</td>
<td>175</td>
</tr>
<tr>
<td>8.1 Conclusions</td>
<td>175</td>
</tr>
<tr>
<td>8.2 Future Work</td>
<td>178</td>
</tr>
<tr>
<td>List of References</td>
<td>181</td>
</tr>
<tr>
<td>Appendix A</td>
<td></td>
</tr>
<tr>
<td>Protocols for Cell Culture and Immunolabeling</td>
<td>185</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Listing of the means, medians, modes, standard deviations (SD), coefficient of variation (CV), and the sample size (N) of the magnetized QSC bead magnetophoretic mobility.</td>
<td>23</td>
</tr>
<tr>
<td>2.2</td>
<td>Results of a reproducibility study of the magnetized Simply Cellular beads on two different days using two different lots of the magnetizing reagent (anti-FITC MACS microbead).</td>
<td>29</td>
</tr>
<tr>
<td>3.1</td>
<td>Listing of the means, medians, modes, standard deviations, and coefficients of variation (CV) for the magnetophoretic mobility measurements of immunomagnetically labeled fibrosarcoma populations. Also included are number of cells analyzed for each population group.</td>
<td>50</td>
</tr>
<tr>
<td>3.2</td>
<td>Listing of the mean magnetophoretic mobility measurements, mean cell diameters of the different fibrosarcoma treatment populations, the slope, adjusted for the differences in drag force due to differences in the mean cell sizes, and the calculated mean antibody binding capacity for each cell treatment population.</td>
<td>56</td>
</tr>
<tr>
<td>3.3</td>
<td>Listing of the coefficients of variation (CVs) for the two types of analyses, fluorescent intensity measurements from FCM and magnetophoretic mobility measurements from CTV, for QSC microbeads and fibrosarcomas.</td>
<td>62</td>
</tr>
<tr>
<td>5.1</td>
<td>Table listing the magnet susceptibilities of paramagnetic and diamagnetic elements and inorganic compounds.</td>
<td>121</td>
</tr>
<tr>
<td>7.1</td>
<td>List of results from FCM analysis of antigens on hematopoietic cells.</td>
<td>171</td>
</tr>
<tr>
<td>7.2</td>
<td>Table listing the separation results of apheresis product immunomagnetically labeled with antibody reagent in MACS CD34 Isolation Kit.</td>
<td>172</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Diagram of immunomagnetic labeling of Quantum Simply Cellular® (QSC) microbeads</td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>Diagram of amplification of immunomagnetic particle binding using three magnetic microbeads per one antigen site as an example</td>
<td>9</td>
</tr>
<tr>
<td>2.3</td>
<td>Diagram of CTV experimental set-up</td>
<td>18</td>
</tr>
<tr>
<td>2.4</td>
<td>Histograms of the magnetophoretic mobilities of the immunomagnetically labeled Quantum Simply Cellular microbead populations</td>
<td>20</td>
</tr>
<tr>
<td>2.5</td>
<td>Histograms of magnetophoretic mobilities of the controls for Quantum Simply Cellular microbeads</td>
<td>21</td>
</tr>
<tr>
<td>2.6</td>
<td>Plot of the magnetophoretic mobilities versus the given antibody binding capacities of the immunomagnetically labeled Quantum Simply Cellular microbeads</td>
<td>25</td>
</tr>
<tr>
<td>2.7</td>
<td>Histograms of immunomagnetically labeled Simply Cellular microbeads with identical and different lots of MACS paramagnetic antibody labels</td>
<td>28</td>
</tr>
<tr>
<td>3.1</td>
<td>Schematic of an immunomagnetically labeled fibrosarcoma cell</td>
<td>44</td>
</tr>
<tr>
<td>3.2</td>
<td>Histograms of the magnetophoretic mobilities of the immunomagnetically-labeled 0-hour, 12-hour, and 24-hour IFN treatment cell populations along with the unlabeled cells and non-specifically labeled cells</td>
<td>49</td>
</tr>
<tr>
<td>3.3</td>
<td>Linear plot of the magnetophoretic mobilities, as measured by CTV, and the fluorescent intensities, as measured by FCM, for different IFN treatment populations of fibrosarcoma cells</td>
<td>51</td>
</tr>
<tr>
<td>3.4</td>
<td>Histogram of QSC microbead diameter measurements</td>
<td>53</td>
</tr>
</tbody>
</table>
3.5 Histogram of diameter measurements of untreated (0-hour IFN treatment) fibrosarcoma cells ................................................................. 54

3.6 Semi-log plot of the estimated maximum number of MACS nanobeads (approximately 30nm, 50nm, and 100nm in diameter) and corresponding ABC, assuming $\beta=4$, to a cell or microbead surface versus size of that cell or microbead ............................................................................................................. 58

3.7 Histograms of fluorescence intensities of the QSC microbeads.............. 60

3.8 Linear plot of the median fluorescent intensities of the QSC microbead versus ABC ................................................................. 61

3.9 Histograms depicting the fluorescence intensities of the immunofluorescently labeled and unlabeled fibrosarcoma cells, untreated (0-hour IFN treatment) ....................................................................... 61

4.1 T-cells labeled with four different immunomagnetic antibody labeling Schemes ................................................................................................. 79

4.2 Cellular fluorescence intensity as a function of the added primary anti-CD4 FITC antibody ............................................................................................. 84

4.3 Cellular magnetophoretic mobility as a function of the added secondary anti-FTTC MACS antibody ............................................................................................. 85

4.4 Semi-log histogram showing the fluorescence intensities of peripheral blood mononuclear cells ................................................................................ 90

4.5 Cell size histogram of the white blood cell population separated on a Ficoll cushion ................................................................................ 91

4.6 Cell size histogram of the white blood cell population separated on a Ficoll cushion after monocyte deletion ................................................................................. 92

4.7 Semi-log histogram of the mobilities of the cells labeled with an irrelevant antibody ................................................................................................. 93

4.8 Semi-log histogram of the magnetophoretic mobilities (and corresponding velocities) of the cells labeled with anti-CD4 FITC and anti-FTTC MACS nanoparticles ................................................................................................. 95

4.9 Linear histogram of the magnetophoretic mobilities the cells labeled with anti-CD4 FITC and anti-FTTC MACS nanoparticles ............................................................................................................. 96
4.10 Semi-log histogram of the magnetophoretic mobilities (and corresponding velocities) of the cells labeled with anti-CD4 MACS nanoparticles .......... 97

4.11 Linear histogram of the magnetophoretic mobilities the cells labeled with anti-CD4 MACS nanoparticles ................................................................. 98

4.12 Semi-log histogram of the magnetophoretic mobilities (and corresponding velocities) of the cells labeled with mouse anti-CD4 FITC and anti-mouse MACS nanoparticles ......................................................................................... 99

4.13 Linear histogram of the magnetophoretic mobilities the cells labeled with mouse anti-CD4 FITC and anti-mouse MACS nanoparticles ......................... 100

4.14 Semi-log histogram of the magnetophoretic mobilities (and corresponding velocities) of the cells labeled with FITC conjugated and unconjugated anti-CD4 antibodies and anti-FITC MACS nanoparticles ......................................................... 101

4.15 Linear histogram of the magnetophoretic mobilities the cells labeled with FITC conjugated and unconjugated anti-CD4 antibodies and anti-FITC MACS nanoparticles ......................................................................................... 102

4.16 Linear plot of the median cellular fluorescence intensity versus the corresponding antibody binding capacity of Quantum Simply Cellula calibration microbeads labeled with either FITC conjugated anti-CD4 antibodies and anti-FITC MACS nanoparticles or a mixture of FITC conjugated and unconjugated anti-CD4 antibodies and anti-FITC MACS nanoparticles ......................................................................................... 106

4.17 Semi-log histogram of the magnetophoretic mobilities of magnetic and nonmagnetic polymeric microspheres. ................................................................. 108

4.18 Semi-log histogram of the magnetophoretic mobilities of magnetic and nonmagnetic polymeric microspheres. ................................................................. 109

4.19 Semi-log histogram of the magnetophoretic mobilities of magnetic and nonmagnetic polymeric microspheres ................................................................. 110

5.1 Diagram of an immunomagnetically labeled cell ......................................................... 116

5.2 Diagram of cells immunomagnetically labeled with two different sized magnetic particles ......................................................................................... 120
5.3 Logarithmic plot of the magnetophoretic mobilities over a range of the number of magnetic particles bound to an immunomagnetically labeled cell or microbead for two different magnetic particles: MACS and Immunicon labeled Quantum 25® microbeads ................................................. 122

5.4 Diagram of immunomagnetically labeled cells with different ABC values 123

5.5 Linear plot of magnetophoretic mobility versus the ABC of QSC calibration microbeads ................................................................. 124

5.6 Logarithmic plot of the maximum number of magnetic particles which can bind to a cell (diameter = 7 μm) versus the size of the magnetic particles.... 125

5.7 Diagram of cells immunomagnetically labeled with a\direct and indirect antibody labeling scheme ......................................................... 127

5.8 Linear plot of the number of magnetic particles binding to a cell versus the antibody binding capacity of that cell .............................................. 128

5.9 Logarithmic plot of the magnetophoretic mobilities of a typical immunomagnetically labeled cell population .............................................. 129

5.10 Plot of the relative contributions of the two factors which contribute to the large variation in magnetophoretic mobilities ...................................... 131

6.1 Diagram of experimental set-up for pulse-injecting samples into a quadrupole continuous magnetic cell sorter ........................................... 141

6.2 Schematic showing the separation of magnetic particles into b stream ...... 143

6.3 Histogram of the magnetophoretic mobilities of immunomagnetically labeled QSC microbead populations .................................................. 146

6.4 Theoretical recoveries of immunomagnetically labeled QSC microbeads with median ABC of 16,000 .............................................................. 148

6.5 Theoretical recoveries of immunomagnetically labeled QSC microbeads with median ABC of 53,000 .............................................................. 148

6.6 Theoretical recoveries of immunomagnetically labeled QSC microbeads with median ABC of 210,000 ............................................................ 149

6.7 Experimental and theoretical recoveries of immunomagnetically labeled QSC microbeads with median ABC of 18,000 .............................. 149
7.12 Magnetophoretic mobilities versus ABCs for QSC calibration microbeads immunomagnetically labeled with anti-CD34 FITC and anti-FITC MACS reagents and the antibodies from MACS CD34+ Isolation Kit ............... 173
LIST OF NOMENCLATURE

ABC  Antibody binding capacity
B    Magnetic flux density (tesla)
Dc   Diameter of the cell or microbead (m)
Fb   Magnetic force acting on one paramagnetic nanoparticle (kg-m/s²) = kSₘ
Fbou Buoyancy force (kg-m/s²)
Fd   Drag force (kg-m/s²)
Fg   Gravitational force (kg-m/s²)
Fm   Magnetic force (kg-m/s²)
f    Friction coefficient (kg/s)
k    Constant representing the magnetic property of a single magnetic nanoparticle
m    Magnetophoretic mobility (mm²/T-A-s)
nₙₛ Number of non-specific binding sites per cell
nₛ   Number of specific antigen molecule binding sites per cell
n₁   Number of antigen binding sites per cell
n₂   Number of binding sites on the primary antibody recognized by the secondary antibody
n₃   Number of magnetic nanoparticles conjugated to the secondary antibody
Sₘ   Magnetophoretic driving force (tesla-ampere/mm²) = \( \frac{\nabla B^2}{2\mu_0} \)
Vₘ   Volume of paramagnetic material per paramagnetic nanoparticle (m³)
Vc   Velocity of moving cell or microbead (m/s)
β    Number of magnetic nanoparticles bound each primary antibody
χₘ   Magnetic susceptibility of the magnetic material (SI units)
χₙ   Magnetic susceptibility of the fluid (SI units)
Δχ  Difference between χₘ and χₙ (SI units)
φ    Fraction of binding sites now available due to mixing FITC-conjugated antibody reagents with unconjugated antibodies
η    Viscosity of the suspension fluid (kg/m-s)
λ₁   Valence of primary antibody binding
λ₂   Valence of secondary antibody binding
μ₀   Magnetic permeability of free space (tesla-m/ampere)
θ₁   Fraction of antigen site on the particle surface bound by primary antibody
θ₂   Fraction of sites on the primary antibody bound by the secondary antibody
χₙ   Magnetic susceptibility of the magnetic material (SI units)
ψ    Secondary antibody binding amplification due to secondary antibodies binding to multiple sites on the primary antibody
CHAPTER 1

INTRODUCTION

The ability to separate cells has important applications in the diagnosis and treatment of disease. A current application attracting interest is the separation of hematopoietic progenitor cells from human umbilical cord blood, normal bone marrow, and peripheral blood. These cells are able to reconstitute hematopoiesis in humans and may be used for the treatment of patients having undergone chemotherapy (de Wynter et al., 1995 and Handretinger et al., 1998). Other medical applications for cell separation include the isolation of cancer cells from blood for analysis and an early diagnosis (Racila et al., 1998) and the isolation of fetal cells from blood for DNA testing.

Current, commercially-available cell separation technologies include Fluorescent Activated Cell Sorting (FACS), Mini MACS, Dynabeads, MPC (particulate, magnetic beads), CELLector Flasks (panning technique), and CellPro (avidin-coated beads in a column) (Chalmers et al., 1998a). Of these cell separation systems, FACS is the only method that uses a flow mode of separation, as opposed to batch mode. While batch devices are relatively simple, they have limited throughput volumes and provide little control over the separation process. FACS, however, is expensive and still has relatively slow separation rates.
Currently, state-of-the-art non-batch, high-throughput, continuous immunomagnetic cell sorting devices (quadrupole and dipole immunomagnetic cell sorters) are under development in the laboratories of Jeff Chalmers, The Ohio State University, and Maciej Zborowski, The Cleveland Clinic Foundation (Sun et al., 1998 and Moore et al., 1998). These devices can be used for large-scale isolation of cell populations. Two distinct advantages of continuous immunomagnetic cell sorters are that these magnetic flow sorters are significantly less expensive and potentially orders of magnitude faster than FACS. In addition, magnetic flow sorting does not induce the high cellular shear stresses that are present in FACS separation.

Immunomagnetic separation processes commonly employ paramagnetic nanoparticles conjugated to antibodies directed against specific cellular molecules. Using these paramagnetic nanoparticles, the volume of paramagnetic reagent bound to a cell will dictate that cell’s magnetophoretic mobility when it is placed in an external magnetic energy gradient. In continuous immunomagnetic cell separators, the design and operation are highly dependent on this degree to which a cell is immunomagnetically-labeled. This “degree of immunomagnetic labeling” can be quantitated by obtaining the magnetic susceptibility and the magnetophoretic mobility of the immunomagnetically-labeled cell (Chalmers et al., 1999a and Chalmers et al., 1999b). A technique to determine the magnetophoretic mobility of an immunomagnetically-labeled cell (or particle), on a cell-by-cell-basis, has been developed (Reddy et al., 1998). This technique involves videotaping the movement of immunomagnetically-labeled cells through a medium of known viscosity and magnetic susceptibility in a well-defined magnetic energy density gradient. The velocity of each cell along with its location within the
magnetic energy gradient is recorded. From this information, the magnetophoretic mobility of each cell is obtained. The technique, referred to as Cell Tracking Velocimetry (CTV), now includes semi-automated computer video imaging and tracking analysis (Chalmers et al., 1999a and Chalmers et al., 1999c). The CTV magnet has a region of nearly constant magnetic energy gradient, which greatly simplifies the analysis (Chalmers et al., 1999b).

Since knowledge of the degree of immunomagnetic labeling is a central consideration in the design and operation of continuous immunomagnetic cell separation, an understanding of the antibody binding mechanisms, used to bind paramagnetic material to the cell, is critically important. The work in this thesis provides research studies focused on quantitation of the antibody binding mechanisms involved in immunomagnetically labeling cells. Through the work in this thesis, significant advancements have been made in our understanding of immunomagnetic antibody binding. One of the parameters governing the degree of immunomagnetic labeling and thus, the magnetophoretic mobility of an immunomagnetically labeled cell is called the “antibody binding capacity” or ABC. ABC, related to the number of target cellular antigen molecules, is a term that and provides quantitative information about the number of primary antibodies binding to the surface molecules on individual cells. This work has investigated the proportionality between magnetophoretic mobility and ABC using ABC calibration microbeads. The correlation between magnetophoretic mobility and ABC was then used to quantitate the ABC of a genetically engineered fibrosarcoma model cell line and CD34+ cells from normal human bone marrow, cord blood, and mobilized
peripheral blood from patients undergoing autologous stem cell transplantation. Note that the word quantitate is used here, referring to the quantitation, or quantification, of the number of antigens expressed on a cell.

The amount of paramagnetic material bound to a cell is not only a function of the cell's ABC of the primary antibody, but is also related to the number of secondary antibodies conjugated with paramagnetic material, which bind to epitopes on the primary antibody. In the case where multiple epitopes, or areas of highly specific binding regions, exist on the primary antibody, several secondary antibodies may bind per primary antibody. This amplifies the total amount of paramagnetic material bound to the cell and thus would also amplify the magnetophoretic mobility of the target cells leading to a more pure cell separation. The work in this thesis shows that by careful manipulation of the experimental antibody labeling conditions, it is possible to quantitate the antibody binding amplification of the secondary antibody binding to multiple sites on the primary antibody.

A complicating factor in continuous magnetic cell separation is normal patient-to-patient variation of the ABC of a specific subset of cells. This variation of ABC will change the optimal flow conditions for a separation, thus it is important to understand the relationship between ABC and separation flow rates in order to obtain an optimal separation. Experiments have been conducted aimed to establish the relationship between ABC and magnetic separation efficiency. This relationship was investigated by injecting a suspension of immunomagnetically-labeled Quantum Simply Cellular (QSC)
calibration microbeads into fluid flowing through a quadrupole magnetic sorter and evaluating the elution profiles of the outlet streams using UV detectors for several separation flow rate conditions.

This thesis is written such that each chapter consists of a paper. The work in chapter 2 contains results from some of the experiments from the work for my M.S. degree, but is a significant extension of the mathematical model originally presented in my master's thesis. The magnetophoretic mobility measurements of the fibrosarcoma cells presented in chapter 3 are also data from my master's thesis, but a significant amount of work was added in the calibration of the ABC of these cells and comparison with FCM analysis. Chapters 4, 5, 6 and 7 are completely new and original works and Chapter 8 summarizes the conclusions in these earlier chapters and provides ideas for future research to further develop the ideas presented in this thesis.
CHAPTER 2

MAGNETOPHORETIC MOBILITIES CORRELATE TO ANTIBODY BINDING CAPACITIES

2.1 Introduction

An analogy can be drawn between the magnetophoretic mobilities obtained from the CTV system and the fluorescence intensities obtained from Fluorescent Activated Cell Scanning in Flow Cytometry (FCM). In the CTV system, the measured parameter is the induced velocity of the immunomagnetically-labeled cell when placed within the magnetic energy gradient. In a FCM system, the analogous measured parameter is the fluorescence intensity of an immunofluorescently labeled cell or particle when excited by a laser beam of the appropriate wavelength. However, the induced velocity (or magnetophoretic mobility) in the CTV system is reported on an absolute scale, mm/s (or mm$^3$/T-A-s, millimeter cubed per tesla-ampere-second), while in FCM, the fluorescence intensity signal (FI) is an amplified and relative value that requires significant standardization and control (Schwartz and Fernandez-Repollet, 1993). Also, due to this
absolute nature of magnetic and CTV technology, equations can be developed which model the movement of an immunomagnetically-labeled cell or particle within the magnetic energy gradient.

One parameter of interest governing the measured magnetic or fluorescent signal is called the "antibody binding capacity" or ABC. ABC is a term that provides quantitative information about the number of antibodies binding to the surface molecules on individual cells. To conveniently obtain quantitative information about cell surface molecule expression levels, microbead standards that bind antibodies in a well-calibrated manner are required. Quantum Simply Cellular® Microbeads (Flow Cytometry Standards Corporation, San Juan, Puerto Rico) are one example of such a microbead standard used for FCM. These microbeads have varying capacities to bind mouse monoclonal antibodies (Zagursky et al., 1995). When fluorochrome-conjugated mouse monoclonal antibodies bind to these specific receptors, the microbead's fluorescence intensity signal from FCM is directly proportional to the number of antigen molecules on that microbead or cell labeled with similar antibodies. FCM analysis of immunofluorescently-labeled cells, along with immunofluorescently-labeled microbead standards correlating FI with ABC, allows quantitation of the cells' surface molecule expression levels (Schwartz and Fernandez-Repollet, 1993). In the same way, when paramagnetic material is bound to the antigen sites on this microbead standard, the induced mobility will be directly proportional to the number of surface antigens and thus, ABC.
2.2 Mathematical Model of Magnetophoresis

The equations presented here are modified from previously published work and will provide a new, more thorough attempt to investigate and quantify all of the related parameters that contribute to the magnetically-induced movement of an immunomagnetically-labeled particle (Chalmers et al., 1999b). For a paramagnetically-labeled cell or microbead, the forces impacting its movement through a liquid suspension are magnetic ($F_m$), buoyancy ($F_{bou}$), gravity ($F_g$), and drag ($F_d$) forces. The paramagnetic force acting on an immunomagnetically-labeled cell or microbead using a two-step labeling protocol (Figures 2.1 and 2.2), can be represented as:

$$F_m = (n_1 \theta_1 \lambda_1)(n_2 \theta_2 \lambda_2) \beta_2 F_b$$  \hspace{1cm} (2.1)

where subscripts 1 and 2 refer to the primary and secondary labeling step, respectively; $n_i$ is the number of antigen molecule sites per cell, including specific and non-specific antigen sites ($n_s + n_{ns}$), and $\theta_i$ is the fraction of antigen molecules on the particle surface bound by the primary antibody. The parameter, $\lambda_i$, represents the valence of the primary antibody binding. CD4 antibody binding is bivalent, meaning that one antibody binds two CD4 antigen molecules, thus the value for $\lambda_i$ would be $\frac{1}{2}$ (Davis et al., 1998).

The combined term $n_i \theta_i \lambda_i$ is equivalent to the commonly used term “antibody binding capacity” ($ABC$) of a cell population (Zagursky et al., 1995). Antibody binding capacity is a measure of the number of primary antibodies binding to a cell or microbead.
Figure 2.1: Diagram of immunomagnetic labeling of Quantum Simply Cellular® (QSC) microbeads.

Figure 2.2: Diagram of amplification of immunomagnetic particle binding using three magnetic microbeads per one antigen site as an example (see also Eq 1).
This value includes not only the number of antigen molecules per cell, but also variables such as valence of antibody binding, steric hindrance, binding affinities and non-specific binding. Thus we have:

$$ABC = n_1 \theta_1 \lambda_1$$ \hspace{1cm} (2.2)

The same sequence of parameters is then repeated for the binding of the secondary antibody to sites on the primary antibody. In this case, $n_2$ is the number of binding sites on the primary antibody recognized by the secondary antibody. For example, if the secondary antibody is a mouse anti-fluorescein isothiocyanate (FITC), $n_2$ will be the number of FITC molecules conjugated to that primary antibody. This value is often referred to as the fluorescein-to-protein ratio ($F/P$) of the antibody reagent. $\theta_2$ is the fraction of binding sites on the primary antibodies that are bound by the secondary antibodies and $\lambda_2$ represents the valence of the secondary antibody binding (i.e. the number of secondary antibodies that bind per one FITC molecule). $\beta_2$ is the number of magnetic nanoparticles conjugated to the secondary antibody. The parameters $n_2 \theta_2 \lambda_2 \beta_2$ may be combined into one overall term, $\beta$. In this case, $\beta$ represents the number of magnetic nanoparticles bound to each primary antibody on a cell or microbead. The lumped term $\beta ABC$ describes the number of magnetic nanoparticles bound to each cell or microbead, and is therefore referred to as the “magnetic particle binding capacity” of a cell or microbead.

$F_b$ is the magnetic force acting on one paramagnetic nanoparticle in the direction of the magnetic energy gradient and is described by:
\[ F_b = \frac{1}{2\mu_0} \Delta \chi V_m \nabla B^2 \]  

(2.3)

where \( \mu_0 \) is the magnetic permeability of free space, \( \Delta \chi \) is the difference in magnetic susceptibility between the magnetic material, \( \chi_b \), and the surrounding medium, \( \chi_f \). \( V_m \) is the volume of paramagnetic material per paramagnetic nanoparticle and \( B \) is the magnetic flux density.

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

\[ F_d = 3\pi \eta D_c v_c = f v_c \]  

(2.4)

where \( v_c \) is the velocity of the cell moving through the fluid, \( D_c \) is the diameter of the cell or microbead, \( \eta \) is the viscosity of the fluid, and \( f \) is the friction coefficient of the moving cell or microbead:

\[ f = 3\pi \eta D_c \]  

(2.5)

The following assumptions have been made. It has been assumed that the number of surface molecules expressed on the cell, \( n_t \), is independent of the cell size, or \( D_c \). This assumption is justified by the practice of FCM analysis, in which the cells may be independently gated by their size. Typically, for a given size gating, there is considerable
variation in the cells’ fluorescence intensities (proportional to antigen expression), indicating that there are significant differences in the antigen numbers expressed on cells that are essentially the same size.

However, if, in fact, the cell has an internal regulatory feedback control on the number of surface molecules it expresses per cell membrane surface area, then the number of antigen molecules on the cell surface would be related to the diameter of the cell. Preliminary work, investigating the theoretical effect of cellular size on the velocity (mobility) and cell separation, assumed this dependence, using the term “antigen density”. (Chalmers et al., 1998b). Since this cellular internal feedback control of antigen numbers per membrane surface area is not a consistently true phenomenon, we choose to treat the variables $n_f$ and $D_c$ separately, thus the model may be applied whether antigen expression is a function of cell size or whether it is independent of cell size.

Second, $n_2$, $\theta_2$, $\lambda_2$, $\beta_2$, and thus $\beta$, as well as $\Delta \chi$ and $V_m$, are all independent of ABC, suggesting that the total paramagnetic force acting per immunomagnetically-labeled surface molecule remains the same for all labeled surface molecules. This also requires that the same volume of total paramagnetic material is bound to each antibody and thus to each targeted surface molecule of interest, and that the binding affinities of antibodies to antigens are independent of ABC. The values of $n_2$, $\theta_2$, $\lambda_2$, $\beta_2$, $\Delta \chi$, and $V_m$ may actually vary, but these variations cancel out when averaged over tens of thousands of surface binding sites. The overall effect from each of these parameters will remain the same from cell to cell or particle to particle. Third, drag forces arising from the magnetically
induced velocity of a cell are the only flow effects on the labeled cell or microbead.

Fourth, due to the rather large size of a cell, thermal and Brownian motion are assumed to be negligible. The force balance on the cell or microbead can be written as:

\[ \text{mass} \times \text{acceleration} = F_m + F_d + F_g + F_{\text{bou}} \]  

(2.6)

For micrometer-sized particles, the non-stationary term on the left hand side of the equation is several orders of magnitude smaller than the magnetic and drag forces of fast moving particles, thus, we set the left side of the equation equal to zero (Reddy et al., 1998). Also, since the magnetic energy gradient in the experimental system is perpendicular to gravity, we are only interested in the horizontal movement of the cell or microbead through the fluid. This reduces the force balance equation to:

\[ 0 = F_m - F_d \]  

(2.7)

Substituting Eqs 2.1, 2.2 and 2.4 into Eq 2.7 and noting that magnetic and drag forces are in opposite directions, we obtain a relationship for the magnetic energy gradient induced velocity, \( v_c \), of the moving paramagnetized cell or microbead:

\[ v_c = \frac{(n_1 \theta_1 \lambda_1)(n_2 \theta_2 \lambda_2) \beta_2 |F_b|}{3\pi D_c \eta} = \frac{ABC \beta |F_b|}{f} \]  

(2.8)
where the straight brackets, $| |$, denote magnitude of a vector. The magnetophoretic driving force, $S_m$, is proportional to the magnetic energy gradient and is defined as:

$$S_m = \frac{|\nabla B^2|}{2\mu_0}$$  \hspace{1cm} (2.9)

and magnetophoretic mobility, $m$, is defined as:

$$m = \frac{\nu_c}{S_m}$$  \hspace{1cm} (2.10)

Substituting Eq 2.3 into 2.8 and then Eqs 2.8 and 2.9 into 2.10, we obtain an equation for the magnetophoretic mobility, a "normalized" parameter analogous to electrophoretic mobility, of an immunomagnetically-labeled cell or microbead:

$$m = \frac{\Delta\chi V_m}{3\pi D_c \eta} \beta ABC = \frac{k}{f} \beta ABC$$  \hspace{1cm} (2.11)

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

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

Again, the lumped term, $\beta ABC$, in Eq 2.11 represents the magnetic particle binding capacity of a cell or microbead. Thus, Eq 2.11 concludes that the magnetophoretic mobility of an immunomagnetically-labeled cell or microbead is directly proportional to the number of magnetic nanoparticles binding to the cell or microbead and that the magnetophoretic mobility is inversely proportional to the cell or microbead's friction coefficient. We set out to investigate the predicted proportionality between $m$ and $ABC$.

2.3 Experimental Methods

2.3.1 Quantum Simply Cellular® Microbeads

Quantum Simply Cellular® microbeads (Flow Cytometry Standards, Corp., San Juan, PR) are uniform, 8.5 micron polystyrene microbeads with calibrated numbers of goat anti-mouse antibodies (GAM) bound to their surfaces. Individual sets of these microbeads are coated with four distinct populations of GAM antibodies that bind the Fc region of IgG1, IgG2a, and IgG2b isotypes of mouse monoclonal antibodies and one blank microbead population for controls. Quantum Simply Cellular® microbeads were custom-shipped in individually packaged vials of uniform antibody binding populations (Lot # A03366) having median $ABC$ values of approximately 0, 4,500, 16,000, 52,000, and 210,000. Each vial of these microbeads contains 2 million microbeads per ml.
Simply Cellular® (Flow Cytometry Standards, Corp., San Juan, PR) microbeads are single population GAM coated microbeads having a median $ABC$ of approximately 66,000.

2.3.2 Primary Labeling of Microbeads by Immunofluorescent Antibody

Approximately 1.5 million of these microbeads (750 μl) were placed in microcentrifuge tubes and adjusted to 1.5 million microbeads per ml with PBS. Saturating amounts (15.5 μl) of primary mouse (Isotype IgG2a) anti-CD2 FITC antibodies (CD2 Leu-™5b, Becton Dickinson Immunocytometry Systems, San Jose, CA, Lot # 00199, F/P=4.66) were added to the tubes and incubated for one hour at 7°C. These microbeads were then washed with PBS.

2.3.3 Secondary Labeling of Microbeads by Immunomagnetic Antibody

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

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

2.3.5 The CTV Apparatus

The CTV image analysis system was designed to measure the velocity of the paramagnetically labeled cells or other similarly-sized particles in a well-defined magnetic energy gradient (Figure 2.3). The microbead sample was pumped with a Harvard PhD 2000 Programmable Syringe Pump (Holliston, MA), fitted with a 1 cc syringe, in the negative z-direction through flexible tubing into a 1 mm ID square glass channel. An inverted microscope with a 5X objective was focused on the microbeads in the glass channel at the appropriate region of constant force. Light was supplied to the microscope by a Fiber Lite (Dolan-Jenner, Lawrence, MA) fiber optic light source with a fiber optic cable. The movement of the cells or microbeads in the magnetic energy gradient was videotaped with a 30 Hz Cohu (San Diego, CA) CCD 4915 camera and a
Sony SVO-95000MD video recorder. A more thorough discussion of the apparatus can be found in previous publications (Chalmers et al., 1999a, 1999b, and 1999c).

![Diagram of CTV experimental set-up.](image)

**Figure 2.3**: Diagram of CTV experimental set-up.

### 2.3.6 CTV Analysis

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

Execution of proprietary Borland C++ programs that identify the moving particles and calculate their velocities, allows the velocities for each cell/microbead tracked to be converted into magnetophoretic mobilities using mathematical models. More details are provided in separate publications (Chalmers et al., 1999a, 1999b, and 1999c).

2.4 Results

Histograms of the five ABC populations of the immunomagnetically-labeled Quantum Simply Cellular microbeads are seen in Figure 2.4. This Figure is a composite of five separate experiments with each QSC microbead population. The x-axis represents the magnetophoretic mobility values of each immunomagnetically-labeled microbead population. The y-axis represents the fraction of beads with that specific magnetophoretic mobility. Note that the histograms for each ABC population are distinct and that the mean magnetophoretic mobility for each ABC microbead population increases with increasing ABC values. Histograms of the control studies of Quantum Simply Cellular® microbeads with different combinations of antibody labeling are presented in Figure 2.5. These control studies were conducted on the microbead population with ABC equal to zero.
Figure 2.4: Histograms of the magnetophoretic mobilities of the immunomagnetically labeled Quantum Simply Cellular microbead populations.
Figure 2.5: Histograms of magnetophoretic mobilities of the controls for Quantum Simply Cellular microbeads.
Table 2.1 lists the means, medians, modes, standard deviations and coefficients of variation (CV) of the measured magnetophoretic mobilities, as well as the number of microbeads analyzed in each experiment. These rather large standard deviations for the mobilities for each of the Quantum Simply Cellular microbead populations represent the inherent distribution in the numbers of the surface molecules per microbead as well as additional variation due to microbead sizes, immunolabeling methods, and CTV. The reported standard deviations and CVs are common to both FCM and CTV analysis methods. (The CVs for QSC microbeads using FCM analysis range from 39% to 13%.) We expect a slightly larger CV from CTV than FCM because CTV required a two-step antibody-labeling procedure, whereas FCM only required a one-step antibody-labeling procedure. The lowest CTV signal intensities, however, report significantly larger CVs than the analogous CVs from FCM signals. This is the result of the decreasing mean mobility rather than an increasing standard deviation and is explained by the definition of CV (standard deviation divided by the mean). In CTV, the mean mobilities are nearly zero (approximately 0.001 mm/second) for the slowest or non-moving particles, thus we expect the CVs for the slowest mobilities to be very high. We do not see a large value for the CV of weakly fluorescent cells in FCM because of the inherent non-zero autofluorescence of cells.

Statistical analysis on Quantum Simply Cellular microbeads was conducted using SigmaStat. The results of the Tukey Pairwise Multiple Comparison Test indicate that the differences in the means of each ABC microbead treatment group (Figure 2.4) are all statistically significant ($P<0.05$). The differences in mean mobilities obtained from the
Table 2.1. Listing of the means, medians, modes, standard deviations (SD), coefficient of variation (CV), and the sample size (N) of the magnetized QSC bead magnetophoretic mobility.

<table>
<thead>
<tr>
<th>QSC Population</th>
<th>Mean</th>
<th>Median</th>
<th>Mode</th>
<th>SD</th>
<th>CV</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC=0, no Ab</td>
<td>1.58E-05</td>
<td>6.95E-06</td>
<td>7.50E-06</td>
<td>3.51E-05</td>
<td>221%</td>
<td>145</td>
</tr>
<tr>
<td>ABC=0, 2nd Ab</td>
<td>3.25E-05</td>
<td>1.86E-05</td>
<td>1.68E-05</td>
<td>1.01E-04</td>
<td>310%</td>
<td>217</td>
</tr>
<tr>
<td>ABC=0</td>
<td>1.37E-05</td>
<td>1.19E-05</td>
<td>1.19E-05</td>
<td>1.89E-05</td>
<td>140%</td>
<td>163</td>
</tr>
<tr>
<td>ABC=4500</td>
<td>6.01E-05</td>
<td>5.54E-05</td>
<td>5.96E-05</td>
<td>3.54E-05</td>
<td>59%</td>
<td>307</td>
</tr>
<tr>
<td>ABC=16000</td>
<td>2.12E-04</td>
<td>2.09E-04</td>
<td>2.11E-04</td>
<td>7.63E-05</td>
<td>36%</td>
<td>498</td>
</tr>
<tr>
<td>ABC=52000</td>
<td>4.67E-04</td>
<td>4.78E-04</td>
<td>5.31E-04</td>
<td>1.50E-04</td>
<td>32%</td>
<td>1057</td>
</tr>
<tr>
<td>ABC=210000</td>
<td>7.60E-04</td>
<td>7.67E-04</td>
<td>7.50E-04</td>
<td>1.70E-04</td>
<td>22%</td>
<td>782</td>
</tr>
</tbody>
</table>

control populations (Figure 2.5) were not found to be significant ($P<0.05$). In order to be confident that the mean and median values are accurate, many particles from one population need to be analyzed. CTV usually analyzes up to about 1,000 particles for each sample population. In this way, the means of each sample population have statistical significance.

The mean magnetophoretic mobility of each ABC microbead population was plotted against its corresponding median antibody binding capacity for that population (Figure 2.6). Bars around each mean mobility data point represent the 99% confidence intervals for each population mean. The confidence intervals are narrow, even with rather large
standard deviations, because of the large number of mobility data analyzed, i.e. the relatively large sample size (Moore and McCabe, 1993).

Note that at higher ABC values, the plot in Figure 2.6 exhibits saturation-like behavior. It will be shown that steric hindrance issues become important for microbeads with high numbers of antigen binding sites labeled with these paramagnetic nanoparticles. The calibration plot is linear and follows theory for the lower ABC populations, as expected from Eq 2.11. The slope of the linear portion of the calibration plot is:

\[
\frac{k \beta}{f} = \frac{\Delta x V_m \beta}{3 \pi D \eta} = 1.27 \times 10^{-8} \left[ \text{mm}^3 / \text{T-s} \right] (2.12)
\]

where:

\[
\beta = n_2 \theta_2 \lambda_2 \beta_2 (2.13)
\]

The combination of theory and direct observation of the QSC microbead movement in the magnetic field provides important information about the interaction between the QSC microbead and the bound magnetic nanoparticles. First, it can be assumed that the finite size of the magnetic nanoparticles influence the antibody-nanoparticle binding kinetics by a steric hindrance mechanism, thus causing this deviation from linearity at high ABC values. As the ABC value increases, more magnetic nanoparticles bind to the QSC microbead. Eventually, the space available for these magnetic nanoparticles to bind will be depleted. The average diameter of the QSC microbead is 8.5 \( \mu \)m, surface area of 2.27x10^{-10} m^2. Product information from Miltenyi Biotech (Auburn, CA) indicates that
Figure 2.6: Plot of the magnetophoretic mobilities versus the given antibody binding capacities of the immunomagnetically labeled Quantum Simply Cellular microbeads. The linear portion of the experimental curve (solid line) is described by equation \( m = 1.27 \times 10^{-8} + 9.01 \times 10^{-6} \).
the average diameter of the MACS nanoparticle is approximately 50 nm, cross-sectional area of $1.96 \times 10^{-15}$ m$^2$. The maximum number of nanospheres covering the surface of the QSC microbead is calculated to be 105,000. This calculation assumes that both types of spheres behave like rigid bodies and that the magnetic nanospheres form a densely packed hexagonal lattice on the surface of the QSC microbead (90.7% coverage). Also, the nanospheres form only one layer on the QSC microbead, and that the curvature of the QSC microbead may be neglected. Looking at Figure 2.6 and remembering that the lumped term $\beta ABC$ describes the number of magnetic nanoparticles bound to the cell or microbead, conclusions may be made regarding the value of $\beta$. The deviation from linearity occurs at about 27,000 ABC. If one assumes that point at which the deviation from linearity occurs is when the maximum number of nanoparticles have covered the surface of the QSC microbead, then $\beta = 4$.

The calculated value of $\beta = 4$ allows us to estimate some of the parameters entering into the Eq 2.13. Product information from Becton-Dickinson (San Jose, CA) indicates that the fluorescein-to-protein ($F/P$) ratio for the specific lot of anti-CD2 FITC antibodies used in these experiments is equal to 4.66. In the context of this work, $F/P$ is equivalent to $n_2$, therefore, $n_2 = F/P = 4.66$. Product information from Miltenyi Biotec (Auburn, CA) indicates that $\beta_2 = 1$ for MACS antibody-nanoparticles. Entering the values of $\beta$, $n_2$ and $\beta_2$ into Eq 2.13 we obtain that $\theta_2 \lambda_2 = 0.89$. In other words, our results indicate that, on average, as expected, less than one secondary antibody is binding per epitope on the primary antibody.

Second, the linear portion of the dependence of $m$ on $ABC$ allows one to make inferences about the magnetic properties of the magnetic labeling particles, the MACS
nanoparticles. As discussed above, $\beta = 4$. The microbead's friction coefficient, $f$, is calculated from Eq 2.5 for the known viscosity of the medium, $\eta = 0.001$ kg/m/sec, and the diameter of the QSC microbead, $D_c = 8.5$ microns, which leads to $f = 8.01 \times 10^{-8}$ kg/s. Solving for $k$ in Eq 2.12, a value of $k = \Delta \chi V_m = 2.5 \times 10^{-16}$ mm$^3$ is obtained. The parameter $k$ is intrinsic to the magnetic nanobead and is a constant for a given immunomagnetic label.

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

In addition to the above work, a reproducibility study was conducted using Simply Cellular microbeads immunomagnetically-labeled with identical and different lots of MACS nanobead antibody reagent. Figure 2.7 and Table 2.2 show the histogram and listing of the magnetophoretic mobility results of this study. The CTV data indicates up to a 10% deviation from its averaged magnetophoretic mobility using the same lot of secondary antibody tested on same and different days. If a different lot of secondary antibody is used, the recorded mobilities deviate up to 20% from the averaged value. Results from a Pairwise Multiple Comparison Tukey Test indicate that the differences in these magnetophoretic mobilities are all statistically significant ($P<0.05$) due to the very low spread and high number of microbead data.
Figure 2.7: Histograms of immunomagnetically labeled Simply Cellular microbeads with identical and different lots of MACS paramagnetic antibody labels.
<table>
<thead>
<tr>
<th>Simply Cellular</th>
<th>Mean</th>
<th>Median</th>
<th>Mode</th>
<th>SD</th>
<th>CV</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot#8480 - Day 1</td>
<td>2.77E-04</td>
<td>2.89E-04</td>
<td>2.99E-04</td>
<td>8.79E-05</td>
<td>32%</td>
<td>472</td>
</tr>
<tr>
<td>Lot#8480 - Day 1</td>
<td>3.22E-04</td>
<td>3.28E-04</td>
<td>3.35E-04</td>
<td>7.98E-05</td>
<td>25%</td>
<td>515</td>
</tr>
<tr>
<td>Lot#8480 - Day 2</td>
<td>3.50E-04</td>
<td>3.50E-04</td>
<td>3.76E-04</td>
<td>1.11E-04</td>
<td>32%</td>
<td>875</td>
</tr>
<tr>
<td>Lot#8163 - Day 1</td>
<td>2.67E-04</td>
<td>2.61E-04</td>
<td>2.66E-04</td>
<td>1.18E-04</td>
<td>44%</td>
<td>436</td>
</tr>
</tbody>
</table>

**Table 2.2:** Results of a reproducibility study of the magnetized Simply Cellular beads on two different days using two different lots of the magnetizing reagent (anti-FITC MACS microbead). The nomenclature is the same as that in Table 2.1.

### 2.5 Discussion

A major aim of this study was to demonstrate that a relationship exists between the magnetophoretic mobility of immunomagnetically-labeled calibration microbeads and the ABC of those microbeads. This relationship, defined mathematically in Eq 2.11 and experimentally in Figure 2.6, indicates that it is theoretically possible to measure the value of the ABC, and presumably cell surface antigen expression levels, of similarly immunomagnetically-labeled cell populations.

In addition to developing a relationship between magnetophoretic mobility and ABC, another application of this approach is the experimental measurements of the specific parameters associated with the paramagnetic labels. For example, from the $k$ value obtained from the QSC standard curve, it is possible to predict the amount of
paramagnetic material (magnetite) in each magnetic nanoparticle \((k = \Delta \chi V_m, \text{Eqs. 2.11 and 2.12})\) By knowing the value for the volumetric magnetic susceptibility, \(\Delta \chi\), of pure magnetite, one could solve for the volume of magnetite per paramagnetic-antibody bead complex, \(V_m\). The ability to quantitate \(\Delta \chi\) and \(V_m\) would have a significant impact on the further refinement of immunomagnetic cell separation technology.

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

Another potential advantage of CTV, with respect to FCM, is the stability of the detection system. In FCM, due to shifting of the lasers and detectors and amplification of fluorescent signals, FCM quantitation is only valid if the instrument is recalibrated with these microbead standards each time a cell population is analyzed. Due to the absolute nature of magnetic CTV technology with permanent magnets, there is no need to continuously recalibrate the instrument as long as the antibody-labeling protocol has not been altered. The stability of the CTV magnets has been previously demonstrated (Chalmers et al., 1999b).
The CTV technology was originally designed to measure magnetophoretic mobilities of immunomagnetically-labeled cells for performance optimization of continuous magnetic flow sorters. The batch magnetic separation devices, based on cell capture by magnetic substrates, have not been sensitive to differences in magnetophoretic mobility of the immunomagnetically-labeled cells. For continuous flow sorters, the induced mobility is a fundamental, controlling factor in setting parameters for obtaining a successful separation (Moore et al., 1998).

Magnetophoretic mobility measurements may have broader applications than magnetic separation and cellular antigen quantitation. The magnetophoretic velocity of single erythrocytes has been investigated by artificially inducing the magnetic state of iron, Fe(III), with sodium nitriteFe(III) can then be reduced to Fe(II) using a medium containing glucose (Winoto-Morbach et al., 1995a and 1995b, and Chikov et al., 1993). The reduction of Fe(III) decreases the magnetophoretic mobility of the Fe(III)-rich cells because of the higher magnetic susceptibility of Fe(III) than that of Fe(II). It was proposed by those authors that the diagnosis of patients with glucose-6-phosphate dehydrogenase and reductase deficiency could be made by magnetophoresis.

The long-term applications of using calibration microbeads with CTV technology require further investigations of the correlation between magnetophoretic mobility and ABC. Such a correlation would enable us to accurately predict the conditions for continuous separation processes. In addition, the characteristics of the magnetic particles used as a label (the parameter \( k \)) require further investigations to quantitate the influence of paramagnetic compound on cell motion. It is also desirable to investigate in more...
detail the individual contributions of each parameter entering the Eq 2.1. These are the goals of the current work in our laboratories.

2.6 Acknowledgements

The authors wish to acknowledge the advice from Dr. Abe Schwartz from Flow Cytometry Standards, and a helpful manuscript review by Lee R. Moore.
CHAPTER 3

MEASUREMENT OF CD2 EXPRESSION LEVELS OF IFN-α TREATED FIBROSARCOMAS USING CELL TRACKING VELOCIMETRY

3.1 Introduction

Quantitation of cell surface antigens is a term that describes obtaining the total number of specific antigen molecules expressed on a given cell or cell population. In addition to the phenotypic significance of the expression of a specific antigen on a cell, the expression level (typically measured as antibody binding capacity, or ABC) of specific cell surface antigens is also becoming increasingly important in understanding cell function and the prognosis and diagnosis of disease. (Poncelet et al., 1996, Davis et al., 1998, Lavabre-Betrand et al., 1994 and 1996, Creamer et al., 1996, Liu et al., 1996, Bikoue et al., 1996, Jackson et al., 1990, Lenkei et al., 1998, Hultin et al., 1998, Krause et al., 1996) For example, the relative levels of CD34 surface expression in CD34+ bone marrow cells has been found to correlate with the maturity of hematopoietic progenitor cells. The CD34^{bright} cell populations contain the majority of immature hematopoietic cells while the CD34^{dim} cell populations contain more lineage-committed progenitors. (Krause et al., 1996) In addition, regular cell-associated antigen expression levels have been found either over-expressed or under-expressed in many pathological conditions. For example, researchers have found that the CD38 membrane antigen expression on
cytotoxic (CD8+) T-cells is elevated with increased activation of these cytotoxic T-cells, correlating to the progression of disease in human immunodeficiency virus-positive (HIV+) patients. (Liu et al., 1996, Lenkei et al., 1998, Hultin et al., 1998) Also, the CD10 expression of B-lineage acute lymphoblastic leukemia cells has been found to be 33% to 45% greater when compared with normal fetal bone marrow cells, (Lavabre-Betrand et al., 1994) the elevation of CD64 on neutrophils is believed to be an early indication of sepsis, (Davis et al., 1996) and the p75 chain of the IL-2 receptor on T-cells (also called IL-2Rp75 or CD122) appears to be down-regulated for patients with rheumatic disease. (Creamer et al., 1996)

The most common technique used to obtain quantitative information about cell surface molecule expression levels is to use microbead standards combined with Flow Cytometry (FCM) analysis. (Zagursky et al., 1995, Schwartz et al., 1996 and 1998) Quantum Simply Cellular® Microbeads (Flow Cytometry Standards Corporation, San Juan, Puerto Rico recently purchased by Bangs Laboratories Incorporated, Fishers, Indiana) are one example of such calibration microbeads used for FCM analysis. These microbeads have been designed to bind a specific number of monoclonal mouse antibodies. (Zagursky et al., 1995) Under appropriate conditions, fluorochrome-antibody conjugates bind to specifically targeted surface receptors and the measured fluorescent intensity (FI) from these fluorochrome molecules is directly proportional to the number of receptors (antigen molecules) on that microbead. Consequently, when immunofluorescently labeled cells and calibration microbeads are analyzed using the same antibodies, it is possible to quantitate the number of specific antigens on a cell’s surface.
In addition to using antigen quantitation to discreetly differentiate a cell population, antigen expression level is also an important factor in continuous immunomagnetic separation. (McCloskey et al., 2000, Chalmers et al., 1998b, Sun et al., 1998, Moore et al., 1998) In order to optimize the design of immunomagnetic cell separators, it is desirable to know the degree to which a cell is immunomagnetically labeled. A technique to determine the magnetophoretic mobility, correlating to the antigen expression level, of an immunomagnetically labeled cell or particle has been developed by our laboratories. (McCloskey et al., 2000, Reddy et al., 1998) This technique involves videotaping the movement of immunomagnetically labeled cells through a medium of known viscosity in a well-defined magnetic energy gradient. The velocity of each cell along with its location within the magnetic energy gradient is recorded. From this information, the magnetic susceptibility of each cell is obtained. This technique, referred to as Cell Tracking Velocimetry (CTV), now includes semi-automated computer video imaging and tracking analysis. (Chalmers et al., 1999a and 1999c) Also, the CTV magnet has a region of nearly constant magnetic energy gradient, which greatly simplifies the analysis. (Chalmers et al., 1999b) The magnetic energy gradient of this magnet has been demonstrated to be stable over three months, and presumably indefinitely stable, due to the use of permanent magnets. (Nakamura et al., 2000)

An analogy exists between FCM and CTV. In a FCM system, the measured parameter is the fluorescence intensity of an immunofluorescently labeled cell or microbead when excited by a laser beam of the appropriate wavelength. In the CTV system, the measured parameter is the velocity of an immunomagnetically labeled cell or microbead induced by the magnetic energy gradient in which the cells are placed.
However, the induced velocity (magnetophoretic mobility) in the CTV system is reported on an absolute scale, $\text{mm}^3/T\cdot\text{A-s}$ (millimeter cubed per tesla-ampere-second), where in FCM, the fluorescence intensity signal, $\text{FI}$, is an amplified and relative value that requires significant standardization and control. (Zagursky et al., 1995 and Schwartz et al., 1993)

A mathematical model has been developed for magnetic CTV technology that describes all aspects contributing to the movement of an immunomagnetically labeled cell or particle placed in a magnetic energy gradient. (McCloskey et al., 2000) In this previous paper, methodology was also presented which describes using paramagnetically labeled microbeads and CTV technology for antigen quantitation. (McCloskey et al., 2000) Here we have applied the proposed methodology to quantitate the expression levels of CD2 cellular surface antigens on a transformed fibrosarcoma cell line.

### 3.2 Mathematical Model of Magnetophoresis

The more complete derivation of the mathematical theory has been presented in a previous paper. (McCloskey et al., 2000) Here we will summarize the main points of the theoretical development.

The paramagnetic force acting on an immunomagnetically labeled cell or microbead, using a two-step labeling protocol can be represented as:

$$ F_m = (n_1\theta_1\lambda_1)(n_2\theta_2\lambda_2)n_2 F_b $$

(3.1)

where subscripts 1 and 2 refer to the primary and secondary labeling step, respectively; $n_1$ is the number of binding sites per cell, including specific and non-specific binding sites.
(n_s + n_{ns}), \theta_i is the fraction of binding sites on the surface bound by the primary antibody, and the parameter \lambda_i represents the valence of the primary antibody binding.

The combined term n_i \theta_i \lambda_i is equivalent to the commonly used term “antibody binding capacity” (ABC) of a cell population. (Davis et al., 1998) Antibody binding capacity is a measure of the number of primary antibodies binding to a cell or microbead. This value includes not only the number of antigen molecules per cell, but also variables such as valence of antibody binding, steric hindrance, binding affinities and non-specific binding. Thus we have:

$$ABC = n_i \theta_i \lambda_i$$  \hspace{1cm} (3.2)

The same sequence of parameters is repeated for the binding of the secondary antibody to sites on the primary antibody. In this case, n_2 is the number of binding sites on the primary antibody recognized by the secondary antibody, \theta_2 is the fraction of binding sites on the primary antibodies that are bound by the secondary antibodies, \lambda_2 represents the valence of the secondary antibody binding, and n_3 is the number of magnetic nanoparticles conjugated to the secondary antibody. The parameters n_2 \theta_2 \lambda_2 n_3 may be combined into one overall term, \beta. In this case, \beta represents the number of magnetic nanoparticles bound to each primary antibody on a cell or microbead. The lumped term ABC\beta describes the number of magnetic nanoparticles bound to each cell or microbead, and is referred to as the “magnetic nanoparticle binding capacity” of a cell or microbead.
The last term in Equation 3.1, $F_b$, represents the magnetic force acting on one paramagnetic nanoparticle in the direction of the magnetic energy gradient and is described by:

$$F_b = \frac{I}{2\mu_0} \Delta \chi V_m \nabla B^2$$  \hspace{1cm} (3.3)$$

where $\mu_0$ is the magnetic permeability of free space, $\Delta \chi$ is the difference in magnetic susceptibility between the magnetic material, $\chi_b$, and the surrounding medium, $\chi_f$. $V_m$ is the volume of paramagnetic material per paramagnetic nanoparticle and $B$ is the magnetic flux density.

These equations are combined in a force balance where the magnetically-induced forces and drag forces are in opposite directions, perpendicular to gravity, thus a relationship for the induced velocity, $v_c$, of the moving paramagnetized cell or microbead is obtained:

$$v_c = \frac{(n_j \theta_j \lambda_j)(n_j \theta_j \lambda_j) n_j |F_b|}{3\pi \eta D_c} = \frac{ABC \beta |F_b|}{f}$$  \hspace{1cm} (3.4)$$

where $v_c$ is the velocity of the cell moving through the fluid, $D_c$ is the diameter of the cell or microbead, $\eta$ is the viscosity of the fluid, and $f$ is the friction coefficient of the moving cell or microbead. The magnetophoretic mobility, $m$, a “normalized” parameter analogous to electrophoretic mobility, is obtained by dividing the velocity term by the magnetic energy gradient.
\[ m = \frac{v_m}{\left| \nabla B^2 \right|} = \frac{\Delta x V_m \beta}{3\pi \eta D_c} \frac{ABC}{2\mu_0 f} = \frac{k \beta}{f} ABC \quad (3.5) \]

Note that the magnetophoretic mobility is independent of magnetic field strength. Also, 
\( k = \Delta x V_m \) is a constant representing the magnetic property of a single magnetic 
nanoparticle. Again, the lumped term \( ABC\beta \) represents the magnetic particle binding 
capacity of a cell or microbead.

In Chapter 2, it was experimentally demonstrated that the magnetophoretic mobility is 
a function of \( ABC \) for Quantum Simply Cellular microbeads up to approximately 
210,000, and linearly proportional to \( ABC \) up to 30,000. (McCloskey et al., 2000) Figure 
2.6 was a plot of the measured magnetophoretic mobility versus \( ABC \). In this chapter we 
will present the methodology and experimental results in which we attempt to 
approximate the \( ABC \) of a genetically engineered fibrosarcoma cell line using the linear 
portion of the calibration curve in Figure 2.6. We will also outline the current 
assumptions, limitations, and potential solutions involved in obtaining accurate \( ABC \) 
measurements.

### 3.3 Experimental Methods

#### 3.3.1 Fibrosarcoma Cell Line and Culturing Conditions

The cells used for antigen quantification experiments consisted of a human sarcoma 
2C4 cell line. This cell line was derived by stable co-transfection of human fibrosarcoma
HT-1080 cells with pDW 9-27 CD2 and pTK-Neo vectors (thymidine kinase-neomycin). (Watling et al., 1993) Upon treatment of 2C4 cells with an interferon cytokine, these cells send signals via the Jak/STAT pathway to start production and express the CD2 protein molecule on their surface (normally expressed on T-cells). This immortal cell line was kindly provided by George R. Stark, Ph.D., The Lerner Research Institute/NCI 1, Cleveland Clinic Foundation in October 1997 and again in January 2000.

Fibrosarcoma cells were grown to confluence in T-75 tissue culture flasks and split as soon as they grew to confluence. When split, the re-seeding culture concentration was 1/2 of the concentration of a confluent flask. The cells were washed with calcium and magnesium free phosphate buffer solution (PBS). Trypsin-EDTA (Life Technologies Inc., Grand Island, NY) was used as the trypsinizing agent. Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Calf Serum and 1X Penicillin-Streptomycin (all three from Life Technologies Inc.) served as culture medium.

### 3.3.2 Treating Cells with IFN-alpha

The “recombinant leukocyte A interferon” used for these experiments was kindly provided by Hoffman-La Roche Inc., Nutley, NJ (for experimental use only). Before treating fibrosarcoma cells with interferon-α (IFN), the cells were grown to confluence in T-75 tissue culture flasks, and split down to a 1/2 re-seeding concentration 24 hours prior to interferon treatment. The cells were allowed to grow back to confluence, or close to confluence, for the next 24 hours, at which time, a sufficient quantity of the 5 million units per ml of IFN solution was added to 12 ml of culture media to obtain a final
concentration of 1500 units per ml. These cells were then incubated with the IFN treated media for a predetermined number of hours at 37°C.

3.3.3 Cell Harvesting

Treated cells were washed with PBS and given Versene (PBS and 5mM EDTA, Life Technologies Inc.) and incubated for 5 to 10 minutes at 37°C to lift cells off the bottom of the flask. After this incubation, Fetal Calf Serum was added. These cells were then centrifuged, washed, and resuspended in PBS.

3.3.4 Cell Size Analysis

The cells were cultured, treated with IFN, and harvested according to the protocols described above. They were then analyzed with a Coulter Counter Multisizer II®.

3.3.5 Antibody Saturation Studies

Experiments were conducted to insure that the concentration of antibody reagent used in immunolabeling cells was saturating all of the available antigen binding sites. To insure this, the 24-hour IFN-treated cell population (the cell population believed to express the greatest number of antigen binding sites) was incubated with different volumes of FITC-conjugated antibody following the protocols below. Since the primary antibody was an antibody-FITC conjugate, FCM analysis was used for the saturation study of the primary antibody. The CD2 immunofluorescently tagged antigen sites were
assumed to be saturated when the fluorescent intensity signal produced little increase in FI with increasing concentration of FITC conjugated antibody reagent, as suggested in the literature. (Carter et al., 1990)

To check for saturation of the primary antibody, five different volumes (100, 111, 140, 200, and 250 μl) of the primary mouse (Isotype IgG2a) anti-CD2 FITC antibody reagent (CD2 Leu-™5b, Becton Dickinson Immunocytometry Systems, San Jose, CA) were tested on the 24-hour treated cells. The mean fluorescence intensities of these cells were approximately equivalent, FI = 80-95, on a four-decade log scale (FCM was a Beckman Coulter Elite) with a mean autofluorescent intensity of FI = 0.4 for these cells.

Once saturation of the primary antibody was verified, the 24-hour IFN-treated cells were immunofluorescently tagged with anti-CD2 FITC primary antibody followed by incubated with different volumes (60, 75, and 100 μl) of the secondary mouse (Isotype IgG1) anti-FITC MACS antibody reagent (Miltenyi Biotec, Auburn, CA). The mean magnetophoretic mobilities, as measured by CTV analysis, of all three volumes were approximately equivalent ranging from 5.5x10^{-4} to 5.8x10^{-4} mm³/T-A-s while the unlabeled control cells exhibited a mean mobility equal to 1x10^{-5} mm³/T-A-s.

3.3.6 Immunofluorescent Labeling the Fibrosarcoma Cells for FCM Analysis

The IFN-treated and harvested cells were placed in tubes of approximately 7 million cells each and adjusted to a concentration of 1.5 million cells per ml with PBS. Cells were blocked by adding 67 μl of 3 mg/ml of goat IgG (Sigma, Saint Louis, MO) and allowed to incubate for 15 minutes at 7°C. After this incubation, 140 μl anti-CD2 FITC
(lot #80523) was added to the tubes and allowed to incubate for one hour at 7°C. Cells were then washed with 3-5 ml of PBS and then taken for FCM (Beckman Coulter Elite) analysis.

3.3.7 Immunomagnetic Labeling the Fibrosarcoma Cells for CTV Analysis

The IFN-treated harvested cells were labeled according to the protocols above. It was determined that the IgG blocker was not necessary for these magnetically labeled cells (data not shown), so the IgG blocker was left out of the labeling procedure for CTV analysis. After the cells were incubated with the fluorescent antibody, they were resuspended in 5 ml of PBS and labeled with 80 µl of the secondary mouse anti-FITC MACS antibody. Cells treated with IFN for 0, 24, and 48 hours were labeled with mouse anti-FITC MACS antibody reagent from lot #NE5505, while the cells treated with IFN for 12 and 36 were labeled with mouse anti-FITC MACS antibody reagent from a different lot #NE5557. This incubation lasted 30 minutes at 7°C, after which the cells were washed twice with 3-5 ml of PBS. The cells, now ready for CTV analysis, were resuspended in PBS with 10% 20mM EDTA solution so that a concentration of approximately 1-2 million cells per ml was obtained. Figure 3.1 depicts the fibrosarcoma cell with both primary and secondary antibody labels.
Figure 3.1: Schematic of an immunomagnetically labeled fibrosarcoma cell.

3.3.8 Immunofluorescent Labeling the Fibrosarcoma Cells for CD2 Quantitation using FCM

This later FCM study was included to provide an independent calculation of the antibody binding capacity for untreated fibrosarcoma cells. It should be noted that these FCM experiments were conducted many months after the initial experiments for CD2 quantitation and more importantly, the FCM used in this later study was a FACScan rather than the earlier Beckman Coulter Elite. Since the FI values on these two instruments were very different, FI values ranged from 0.1 to 1,000 on the Beckman
Coulter Elite, whereas the FI values on the FACScan ranged from 1 to 10,000, thus the FI values cannot be compared between the two instruments.

The cells were cultured and harvested in the same manner as previously described. The labeling method for this later study included a human IgG blocker rather than the goat IgG blocker. Approximately 4 million of the harvested cells (without IFN treatment) were placed in a tube and adjusted to 2 million cells per ml with PBS. Cells were incubated with 25 μl of human IgG blocking reagent (Miltenyi Biotec, Auburn, CA, lot# NE6846) and a saturating amount, 100 μl, of mouse (Isotype IgG2a) anti-CD2 FITC antibody reagent (CD2 Leu™5b, lot #12609) was then added to the tubes and incubated for 30 minutes at 7°C with mixing every 15 minutes. Cells were then washed twice with PBS, resuspended in 300 μl of PBS and taken for FCM (FACScan, Becton Dickinson, San Jose, CA) analysis.

3.3.9 Quantum Simply Cellular® Microbeads

The Quantum Simply Cellular® (QSC) microbeads used in this study were uniform, 8.0 micron polystyrene microbeads with calibrated numbers of goat anti-mouse antibodies (GAM) bound to their surfaces. Individual sets of these microbeads are coated with four distinct populations of GAM antibodies that bind the Fc region of IgG1, IgG2a, and IgG2b isotypes of mouse monoclonal antibodies and one blank microbead population for controls. The QSC microbeads used in this study were shipped in one vial (lot #A050699) having uniform antibody binding populations of median ABC of approximately 0, 5,500, 18,000, 51,000, and 150,000.
3.3.10 Immunofluorescent Labeling the Microbeads for CD2 Quantitation using FCM

Approximately 100 μl of the QSC microbead suspension and 100 μl of PBS were placed together in a 15 ml centrifuge tube. Four different volumes (20, 40, 80, 100 μl) of the primary mouse (Isotype IgG2a) anti-CD2 FITC (CD2 Leu-™5b) antibody reagent were tested on the microbeads to ensure saturation. The mean fluorescence intensities of the 80 μl and 100 μl volumes were approximately equivalent. Subsequently, the QSC calibration microbeads were labeled with 80 μl of primary mouse (Isotype IgG2a) anti-CD2 FITC antibodies (lot # 12609) and incubated for one hour at room temperature. These microbeads were then washed twice with 2 ml of PBS, resuspended in 300 μl of PBS and analyzed immediately.

3.3.11 The CTV Apparatus

The CTV image analysis system was designed to measure the velocity of the paramagnetically labeled cells or other similarly sized particles in a well-defined magnetic energy gradient, depicted in Figure 2.3. The sample was pumped with a Harvard PhD 2000 Programmable Syringe Pump (Holliston, MA), fitted with a 1 cc syringe, in the negative z-direction through flexible tubing into a 1 mm ID square glass channel. An inverted microscope with a 5X microscope objective was focused on the microbeads in the glass channel at the appropriate region of constant force. Light was supplied to the microscope by a Fiber Lite (Dolan-Jenner, Lawrence, MA) fiber optic
light source with a fiber optic cable. The movement of the cells or microbeads in the magnetic energy gradient was videotaped with a 30 Hz Cohu (San Diego, CA) CCD 4915 camera and a Sony SVO-95000MD video recorder. A more thorough discussion of the apparatus can be found in previous publications. (Chalmers et al., 1999a, 1999b, and 1999c, Nakamura et al., 2000)

3.3.12 CTV Analysis

Analog VCR images from videotapes were converted into 624 X 450 pixel images in which each pixel was assigned a gray-level ranging from 0 (black) to 255 (white or brightest) with a µTech image board (Mutech Corp., Billerica, MA) and M-Vision 1000 Sequence Software (Mutech Corp., Billerica, MA). This software allows the user to adjust gain and offset settings to improve image quality before recording and saving images from the videotape or directly from the camera and converting them into pixel (digital) form. The software also allows the user to choose how many total frames of images to save as well as how many frames to skip before saving the next image. This frame-skipping feature is an important tool that can sometimes significantly reduce noise in the final velocity data. (Nakamura et al., 2000)

Execution of proprietary Borland C++ programs that identify the moving particles and calculate their velocities allow the velocities for each cell/microbead tracked to be converted into magnetophoretic mobilities using mathematical models. More details are provided in separate publications. (Chalmers et al., 1999a, 1999b, 1999c)
3.4 Results

Histogram composites of the magnetophoretic mobilities of five separately analyzed fibrosarcoma cell populations incubated with IFN for 0, 13, and 24 hours along with controls including unlabeled cells and cells labeled with only the secondary antibody, non-specific for cellular antigen site, are presented in the semi-log plot in Figure 3.2. The abscissa represents the magnetophoretic mobility values of individual cells. The ordinate represents the fraction of cells with that specific magnetophoretic mobility. Note that the histograms for each cell population treatment group are distinct and that the mean magnetophoretic mobility increases with increasing IFN incubation periods from 0 to 24 hours. Note also that the untreated cells, 0 hours of IFN treatment, exhibit a distinct mobility significantly greater than that of the controls. This indicates that the cells express CD2 antigen inherently, possibly due to the presence of IFN in the culture serum or a low level of constitutive expression.

Table 3.1 lists the means, medians, modes, standard deviations, and coefficients of variation (CV) for the magnetophoretic mobility measurements, and the number of cells analyzed for each experiment. The variance in the mobility measurements represent the inherent distribution in the numbers of the surface molecules per cell, as well as the inherent distribution of cell sizes within a given cell population (Equation 3.4). Additional sources of variation include immunolabeling techniques and CTV experimental analysis methods. The large standard deviations and CVs are common to both FCM and CTV analysis methods for biological systems. In order to be confident that the mean and median values are accurate, many cells from one population have been analyzed. Results from a Pairwise Multiple Comparsion Tukey Test indicate that the
differences in magnetophoretic mobilities of all fibrosarcoma treatment groups, including the unlabeled cells and the cells labeled with the secondary antibody only, are statistically significant (P <0.05).

Figure 3.2: Histograms of the magnetophoretic mobilities of the immunomagnetically-labeled 0-hour, 12-hour, and 24-hour IFN treatment cell populations along with the unlabeled cells and non-specifically labeled cells.
Table 3.1: Listing of the means, medians, modes, standard deviations, and coefficients of variation (CV) for the magnetophoretic mobility measurements of immunomagnetically labeled fibrosarcoma populations. Also included are number of cells analyzed for each population group.

<table>
<thead>
<tr>
<th>IFN Treatment</th>
<th>m [mm$^3$/T-A-s]</th>
<th>Mean</th>
<th>Median</th>
<th>Mode</th>
<th>SD</th>
<th>CV</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells only</td>
<td>1.66E-05</td>
<td>5.70E-06</td>
<td>7.50E-06</td>
<td>4.94E-05</td>
<td>299%</td>
<td>513</td>
<td></td>
</tr>
<tr>
<td>2nd Ab only</td>
<td>4.42E-05</td>
<td>1.83E-05</td>
<td>2.11E-05</td>
<td>9.73E-05</td>
<td>230%</td>
<td>350</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.61E-04</td>
<td>1.30E-04</td>
<td>1.50E-04</td>
<td>1.59E-04</td>
<td>99%</td>
<td>1129</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>3.34E-04</td>
<td>2.98E-04</td>
<td>2.99E-04</td>
<td>2.16E-04</td>
<td>65%</td>
<td>893</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>7.31E-04</td>
<td>5.88E-04</td>
<td>5.96E-04</td>
<td>7.15E-04</td>
<td>100%</td>
<td>1279</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>5.65E-04</td>
<td>5.02E-04</td>
<td>5.31E-04</td>
<td>4.07E-04</td>
<td>77%</td>
<td>1051</td>
<td></td>
</tr>
<tr>
<td>48</td>
<td>6.31E-04</td>
<td>4.66E-04</td>
<td>4.73E-04</td>
<td>6.63E-04</td>
<td>109%</td>
<td>1663</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.3 is a linear plot of the mean magnetophoretic mobilities from CTV analysis of the 0, 12, 24, 36, and 48-hour IFN-treated cell populations, and a plot of the mean linear fluorescence intensities from FCM analysis (Beckman Coulter Elite) of the same 0, 12, 24, 36, and 48-hour IFN-treated cell populations. The error bars around each data point are the 99% confidence intervals for the mean of each sample population. These confidence intervals are rather small, even though the variance for the data is large, due to the very large number of cells analyzed in each sample population. (Moore and McCabe, 1993) The confidence intervals for the fluorescence intensity data are smaller than the confidence intervals for the magnetophoretic mobility data, as expected since FCM measures approximately 10,000 cells, where CTV measures approximately 1,000 cells. The plots in Figure 3.3 clearly show that both techniques, FCM and CTV, can measure relative changes in signal intensities related to CD2 expression levels. We see
that the CD2 levels on fibrosarcoma cells increase with increasing hours of treatment with interferon to up to 24 hours of treatment. Of particular interest is that the magnetophoretic mobilities measured from CTV have absolute units, whereas, the signals from FCM are unitless values.

Figure 3.3: Linear plot of the magnetophoretic mobilities, as measured by CTV, and the fluorescent intensities, as measured by FCM, for different IFN treatment populations of fibrosarcoma cells.

At first glance, one may note that the magnetophoretic mobility curve has pronounced inflection points at 12 and 36 hours, unlike the curve depicting mean fluorescence intensities. After a more complete analysis of the experimental methods, we observed that the lot of anti-FITC MACS secondary antibody reagent used for the 0, 24, and 48 hour treatment populations differed from the lot of anti-FITC MACS antibody reagent.
used for the 12 and 36 hour treatment populations, and the same lot of antibody was used on all treatment groups for FCM analysis. Previous studies reported up to a 20% deviation in mobility measurements between different two lots of anti-FITC MACS antibody reagent. (McCloskey et al., 2000) Since the intensity of the fluorescence signal is directly proportional to the number of FITC molecules, a similar phenomena could possibly be observed in FCM if different lots of primary antibody had been used since the fluorescein-to-protein (F/P) ratio is also known to vary between antibody lots. (Zagursky et al., 1995)

When a cell is immunomagnetically labeled, the size of that cell or microbead has a significant impact on the resulting magnetophoretic mobility. There are two primary aspects in which the size of a cell contributes to the resulting magnetophoretic mobility. The first aspect is more obvious and can be readily observed from the mathematical model (Equation 3.5). Here we see that the drag force on the cell or microbead moving through viscous media is proportional to the diameter of the immunomagnetically labeled cell and, thus the magnetophoretic mobility is inversely proportional to the cell diameter. The second, less obvious aspect, is the potential limitation of the surface area available on the cell or microbead to bind high numbers of nano-sized magnetic particles (a steric hindrance limitation). For the above reasons, it is important to consider the particle diameters in magnetophoretic mobility evaluations. Figures 3.4 depicts the size distribution of Quantum Simply Cellular microbeads (used to obtain the calibration plot presented in Figure 1) as measured using a Coulter Multisizer II. The median QSC microbead diameter of 8.5 μm is chosen to be the most representative statistic over the mean value of 9.1 μm (standard deviation of 1.55 μm). This median diameter value, 8.5
\( \mu m \), is also the value reported from the vendor, Flow Cytometry Standards Corporation, as the size of these microbeads for this specific lot of microbeads. The mean diameter for the fibrosarcoma cell populations ranged from approximately 16 to 18 \( \mu m \) as measured using a Coulter Multisizer II. Figure 3.5 depicts the size distribution of the untreated cell population. The mean cell diameter of this population is measured to be 18.12 \( \mu m \), standard deviation of 3.17 \( \mu m \).

**Figure 3.4:** Histogram of QSC microbead diameter measurements.
Figure 3.5: Histogram of diameter measurements of untreated (0-hour IFN treatment) fibrosarcoma cells.
The calibration plot (Figure 2.6) depicting the magnetophoretic mobilities of Quantum Simply Cellular microbeads versus the ABC for each microbead population presents the possibility of using magnetophoretic mobility measurements as a method of quantifying the ABC of a specific cell population. This calibration plot presents the opportunity to potentially quantitate the ABC for the various fibrosarcoma cell populations presented in this manuscript since the same primary and secondary antibodies were used for immunomagnetic labeling both of the cells and the QSC microbeads. However, two complicating factors also exist: the significantly larger size of the fibrosarcoma cells as compared to the QSC microbeads, and the non-linear nature of Figure 2.6 for ABC values greater than 30,000 to 50,000.

As previously published, the linear portion of the calibration curve depicted in Figure 2.6 consists of the following slope:

\[
slope = \frac{\Delta \chi}{3\pi \eta D_c} = \frac{k\beta}{f} = 1.27 \times 10^{-8} \left[ \text{mm}^3 / T - A - \text{sec} \right] \quad (3.6)
\]

where the variables contributing to the slope in this equation are the antibody reagents used in magnetically tagging the microbeads, \( k\beta \), diameter of the cells or microbeads, \( D_c \), and the viscosity, \( \mu \), of the suspending medium. (McCloskey et al., 2000)

Correction for the differences in the drag force, due to differences in the size between the QSC microbeads and the fibrosarcoma cells, is relatively simple. Specifically, the slope (Equation 3.6) is multiplied by the ratio of the QSC microbead diameter, 8.5 microns, to the mean diameter of that fibrosarcoma cell population, e.g. 18.12 microns for the untreated fibrosarcoma cell population (see Table 3.2). Using the ratio of 55
diameters to correct for the differences between QSC microbeads and the fibrosarcoma cell sizes, new slope values are obtained (Table 3.2). These new slope value account for the larger drag force from the larger fibrosarcoma cells.

The magnetophoretic mobility measurements for the IFN-treated fibrosarcoma cell populations can now be used to obtain the predicted antibody binding capacities for each of the cell-treatment populations. These predicted ABC values are also listed in Table 3.2. The calculated mean ABC for the untreated cell population is 27,000 and increases up to a maximum of 110,000 ABC for the 24-hour treatment cell population.

<table>
<thead>
<tr>
<th>Treatment (hours)</th>
<th>Mean m (mm^3/T-A-s)</th>
<th>Cell dia (microns)</th>
<th>Slope (adjusted for dia)</th>
<th>Calculated ABC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.61E-04</td>
<td>18.12</td>
<td>5.96E-09</td>
<td>27,000</td>
</tr>
<tr>
<td>12</td>
<td>3.34E-04</td>
<td>18.05</td>
<td>5.98E-09</td>
<td>56,000</td>
</tr>
<tr>
<td>24</td>
<td>7.31E-04</td>
<td>16.92</td>
<td>6.38E-09</td>
<td>110,000</td>
</tr>
<tr>
<td>36</td>
<td>5.65E-04</td>
<td>16.53</td>
<td>6.53E-09</td>
<td>87,000</td>
</tr>
<tr>
<td>48</td>
<td>6.31E-04</td>
<td>16.16</td>
<td>6.68E-09</td>
<td>94,000</td>
</tr>
</tbody>
</table>

Table 3.2: Listing of the mean magnetophoretic mobility measurements, mean cell diameters of the different fibrosarcoma treatment populations, the slope, adjusted for the differences in drag force due to differences in the mean cell sizes, and the calculated mean antibody binding capacity for each cell treatment population.

The second complicating factor results from the departure from linearity of mobility versus ABC for ABC > 30,000, Figure 2.6. In a previous publication (McCloskey et al., 2000), we suggested that this departure was most likely caused by insufficient space on the surface of the QSC microbead to allow all of the binding sites to be bound by the magnetic nanoparticles (i.e. a steric hindrance mechanism). This steric hindrance
mechanism would limit the secondary antibody (with the magnetic nanoparticle conjugates) from binding to sites on the primary antibody ($\theta_2 < 1$), thus causing the deviation from linearity at high $ABC$ values. This point of deviation from the normally linear relationship can be approximated as a function of the surface area of the cell or microbead, and correspondingly, cell diameter. Figure 3.6 is a semi-log plot of the maximum number of magnetic nanoparticles that are potentially able to bind as a function of the diameter of the cell or microbead. Since the size of the magnetic nanoparticles used in this study has been reported to range from 20 to 100 nanometers in diameter (Kantor et al., 1998) three different nanoparticle sizes were used in the calculations in Figure 3.6, each line representing a different magnetic nanoparticle diameter. The assumptions used to make these calculations are that both the cell or microbead and nanoparticles behave like rigid bodies, the magnetic nanoparticles form a densely packed hexagonal lattice on the surface of the cell (90.7% coverage), only a single layer of nanoparticles form on the cell or microbead surface, and the curvature of the cell or microbead may be neglected. The corresponding $ABC$ values are also shown in Figure 3.6. These $ABC$ values were obtained from the lumped term $\beta ABC$, which describes the number of magnetic nanoparticles bound to the cell or microbead, using an estimated value of $\beta = 4$ (McCloskey et al., 2000). Close inspection of Figure 3.6 indicates that for 50 nm sized nanoparticles binding to an 8.5 $\mu$m microbead, we would expect to reach an $ABC$ of approximately 30,000 before steric hindrance effects will start to dominate. For the fibrosarcoma cells, 16-18 $\mu$m in diameter, we expect to reach an approximate $ABC$ between 93,000 and 120,000 before steric hindrance effects will start to dominate.
1000000
3

Figure 3.6: Semi-log plot of the estimated maximum number of MACS nanobeads (approximately 30nm, 50nm, and 100nm in diameter) and corresponding ABC, assuming β=4, to a cell or microbead surface versus size of that cell or microbead.

Since quantitation of cell surface antigens using FCM analysis has become an acceptable technique to determine cell surface antigen molecule numbers, quantitative FCM was conducted on the untreated (0-hour IFN treatment) cell population in order to compare the ABC value obtained from CTV analysis with the ABC value obtained from FCM analysis. (Zagursky et al., 1995, Schwartz et al., 1998, and Schwartz et al., 1996) The methodology for antigen quantitation using FCM analysis involves labeling the
target cells and the calibration microbeads with a fluorescently-conjugated antibody reagent. Fluorescence intensities of the calibration microbeads from FCM analysis are plotted against the ABC for each microbead population. Once this calibration plot is obtained, the fluorescence intensity of the target cell population is applied to calculate the mean ABC for that cell population.

The linear fluorescence intensities of the immunofluorescently labeled QSC calibration microbeads are depicted in Figure 3.7. Note that the median fluorescence intensity for each microbead population increases with increasing ABC. Figure 3.8 is a linear plot of the median fluorescence intensities of these QSC calibration microbeads versus the median ABC for each microbead population. The regression line in this plot is obtained using only the first four data points because the fluorescence intensity for the cell population of interest lies within this region. Figure 3.9 is a semi-log histogram depicting the unlabeled and labeled untreated (0-hour IFN treatment) fibrosarcoma cell populations. Note that the median autofluorescence for these large, cultured fibrosarcoma cells is large, \( FI = 28 \), compared with the median autofluorescence, \( FI = 3 \), of the blank microbeads in Figure 3.7. Compensation for the larger cellular autofluorescence was accomplished by subtracting the FI for the unlabeled cell population, \( FI = 28 \), from the FI for the labeled cell population, \( FI = 154 \). (Tchikov et al., 1999) This new value, \( FI = 126 \), was then used for ABC determination for this untreated cell population. Using the equation in Figure 3.8, the calculated ABC for the untreated fibrosarcoma cell population is 33,000. This compares favorably with the calculated ABC value of 27,000 from CTV analysis. Note that the above study (including Figures 3.7, 3.8, and 3.9) aimed to determine the ABC for the untreated fibrosarcoma cell
population using FCM analysis was conducted at a later date and, more importantly, on a separate FCM instrument than the FCM results reported in Figure 3.3, thus it is not possible to compare the FI values between Figures 3.7, 3.8, and 3.9 with values from Figure 3.3.

**Figure 3.7:** Histograms of fluorescence intensities of the QSC microbeads. Each peak represents a different population of microbeads with discrete antibody binding capacities.
Figure 3.8: Linear plot of the median fluorescent intensities of the QSC microbead versus ABC.

Figure 3.9: Histograms depicting the fluorescence intensities of the immunofluorescently labeled and unlabeled fibrosarcoma cells, untreated (0-hour IFN treatment).
Table 3.3 lists the coefficients of variation (CV) for the microbead and cell populations used in this study for the two methods of data analysis, CTV and FCM. In general, we see that the CVs from the two methods are very similar. This indicates that the instrumentation noise introduced from CTV and FCM are approximately equal. The major contribution causing signal variation in both CTV and FCM is most likely the inherent spread in diameters and antibody binding capacities (related to antigen expression) for these biological systems. Also, note that for the cells and microbeads with the lowest signal intensities, the CVs measured by CTV are significantly larger than the CVs for the analogous FCM data. The most likely explanation for the larger CVs by CTV results from the definition of CV, standard deviation divided by the mean. In CTV, as the mean mobility of a cell approaches zero, approximately 0.001 mm/second for the slowest or non-moving particles, the CVs will approach infinity. We do not see this same phenomenon in FCM. Since cells are inherently autofluorescent, the FCM signal does not approach zero; consequently, one does not “divide by zero” in calculating the CV of FCM data.

<table>
<thead>
<tr>
<th>QSC Microbeads</th>
<th>CTV</th>
<th>FCM</th>
<th>Fibrosarcomas</th>
<th>IFN Treatment</th>
<th>CTV</th>
<th>FCM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0, no Ab</td>
<td>221%</td>
<td>33%</td>
<td>Cells only, no Ab</td>
<td>299%</td>
<td>82%</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>140%</td>
<td>39%</td>
<td>0 hours</td>
<td>99%</td>
<td>98%</td>
<td></td>
</tr>
<tr>
<td>4,500</td>
<td>59%</td>
<td>20%</td>
<td>13 hours</td>
<td>65%</td>
<td>92%</td>
<td></td>
</tr>
<tr>
<td>16,000</td>
<td>36%</td>
<td>17%</td>
<td>24 hours</td>
<td>100%</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>52,000</td>
<td>32%</td>
<td>15%</td>
<td>36 hours</td>
<td>77%</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>210,000</td>
<td>22%</td>
<td>13%</td>
<td>48 hours</td>
<td>109%</td>
<td>100%</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3: Listing of the coefficients of variation (CVs) for the two types of analyses, fluorescent intensity measurements from FCM and magnetophoretic mobility measurements from CTV, for QSC microbeads and fibrosarcomas.
3.5 Discussion

The major aim of this study was to demonstrate that in addition to FCM analysis methodology, CTV technology, which measured the magnetophoretic mobility of an immunomagnetically-labeled cell, also has the potential to quantitate cellular antibody binding capacities. This potential was demonstrated by calculating the mean ABC values for the untreated fibrosarcoma cell populations. These ABC values were calculated to equal 27,000 from CTV analysis and 33,000 using FCM analysis. In addition, both CTV mobility measurements and FCM fluorescent intensity measurements qualitatively indicated elevated ABC levels, correlating to CD2 expression levels, for the IFN-treated fibrosarcoma cells (Figure 3.3).

However, at this stage of CTV development and immunomagnetic labeling methods, these results demonstrate only preliminary attempts to quantitatively correlate magnetophoretic mobility to antigen expression levels. To date, we have identified three major challenges in using CTV to quantitate cellular antigen expression levels. These challenges include: 1) the possible steric hindrance of magnetic nanoparticles binding to the limited space available on the cellular surface 2) the limited information available on the precise immunochemical behavior of our antibody reagents, and 3) the current lack of a direct calibration method correlating magnetophoretic mobility to ABC.

As a result of our first challenge, the potential steric hindrance effects due to magnetic nanoparticle binding, we are less confident in our calculations of the mean ABC values for the IFN-treated fibrosarcoma cell populations. These calculated ABC values
for the IFN-treated fibrosarcomas, listed in Table 3.2, assumed that $\beta = 4$, as previously shown. (McCloskey et al., 2000) However, this prediction was made using the linear portion of the calibration curve in Figure 2.6. Remembering that the term $\beta$ was defined as the lumped parameters $n_2 \theta_2 \lambda_2 n_3$, where $\theta_2$ was defined as the fraction of binding sites on the primary antibodies that are bound by the secondary antibodies, we see that $\theta_2$ will decrease when steric hindrance becomes a factor, therefore $\beta$ will also decrease. Within this sterically hindered region, the exact value for $\theta_2$, and thus $\beta$, is not known. Without concrete value for $\beta$, the calculations for cellular ABCs become much more difficult. In future work, it may be possible to circumvent this problem of steric hindrance by mixing FITC conjugated primary antibodies with pure, non-conjugated antibodies. The secondary antibody would have fewer binding sites, thus allowing a larger ABC range for linear calibration and analysis. However, this mixing of antibodies would significantly lower the magnetophoretic mobility of these labeled cells, therefore making them more difficult to separate from non-magnetically labeled cells. For now, we have chosen to focus on the untreated cell population, since these cells express a low enough ABC to escape problems associated with steric hindrance.

When attempting to quantitate antibody binding, it is also important to understand the immunochemical behavior (binding affinities, disassociation constants, non-specific binding, etc.) of the antibody reagents. This information would allow accurate determination of the required amounts of antibody needed to guarantee antibody saturation of the specific binding sites, while also minimizing non-specific binding. In this study, only relatively primitive antibody saturation experiments were conducted according to a currently accepted technique. This involved adding increasing amounts of
antibody reagent until the additional antibody reagent produced very little change in FI, and in our case magnetophoretic mobility. (Carter et al., 1990) However, since this technique was designed for the qualitative studies typically conducted on FCM or for binary magnetic cell separations, these studies are not advanced enough when accurate quantitative results are desired. A much more refined understanding of the functional relationships between the antibody, the specific antigen sites, and the non-specific sites is desired. Current investigations in our laboratories are attempting to address these questions.

In addition to the optimization and evaluation of the antibody reagents, either all of the terms in Equation 5, or the values of “lumped” terms, such as $k$, $\beta$, and $f$, are also needed for ABC quantitation. Values for these “lumped” terms have been experimentally determined using QSC calibration microbeads. (McCloskey et al., 2000) However, due to significant differences between QSC microbeads and cells, the values for these terms also need to be determined independently for the cells. In this study, the ABC of the untreated fibrosarcoma cells was independently obtained using quantitative FCM analysis. Unfortunately, these fibrosarcoma cells have a high autofluorescence (Figure 8c) compromising our confidence in the calculations of ABC. In addition, since the use of QSC calibration microbeads with FCM are an indirect method for ABC quantitation, any limitations in FCM and QSC microbeads would be compounded with the limitations of CTV. Ultimately, it would be desirable to use radioisotopically-labeled antibodies to directly quantify the ABC of the cells used in a CTV calibration study.

In other laboratories, a study comparing the sensitivity of immunofluorescence and immunomagnetic techniques has been conducted. (Tchikov et al., 1999) These authors
used immunofluorescent and immunomagnetic antibody labeling techniques, changing the primary antibody dilution rate, and compared “positive” cell fraction (binary, on/off type measurements rather than the more sophisticated analog mobility measurements conducted in our laboratories) achieved from both techniques. However, it appears as if these investigators used a 10-fold greater cell sample size for magnetophoretic analysis than for FCM analysis without changing antibody protocols accordingly. This would erroneously point towards FCM as the more sensitive measurement system. Also, the sensitivity of the measurement of a “positive” signal depends highly on the magnetic field strength, i.e. magnetic energy gradients, and the magnetic antibody labels. Thus, if the magnetic energy gradients were poorly designed, it would inaccurately appear as if magnetophoretic analysis is not as sensitive as FCM.

From the results presented Figures 3.2 and 3.3 and also in Table 3.3, it is apparent that CTV is capable of measuring CD2 antigen expression levels with comparable sensitivity to that of FCM. In addition, FCM measures only relative intensity signals, whereas CTV measures mobility signals with absolute units. Table 3.3 verifies that both techniques report data with similar variance, indicative from the CVs reported from the two methods of analysis. Additionally, CTV does not have a significant intrinsic mobility signal as seen in FCM autofluorescence signals.

Other researchers have recognized the potential for using magnetophoresis velocity to quantitate numbers of labels per cell, but have chosen to focus on the ratio of magnetophoresis to sedimentation velocity, thus neglecting the dependence of this term on medium viscosity or hydrodynamic properties of cells. (Winoto-Morbach et al., 1995 and 1994, Tchikov et al., 1993) Our work also allows quantitation of the number of
paramagnetic labels per cell, furthermore, includes investigations into antibody binding
capacity and cell diameter as they effect the number of paramagnetic labels one can
attach to a target cell. Since the antibody binding capacity is related to the number of
antigens expressed on a cell, this value may contain information about possible disease
related conditions (Poncelet et al., 1996, Davis et al., 1998, Lavabre-Betrand et al., 1994
and 1996, Creamer et al., 1996, Liu et al., 1996, Bikoue et al., 1996, Jackson et al., 1990,
Lenkei et al., 1998, Hultin et al., 1998), and also provides the structural framework for
antibody paramagnetic nanoparticle binding required to create a magentophoretic
mobility. Current research in our laboratory continues to investigate ABC and other
parameters involved in immunomagnetically labeling a target cell to impart
magnetophoretic mobility for magnetic separation processes. Also, since accurate ABC
values are obtained only when correct concentrations of the labeling antibody reagents
are used, our labs are currently investigating new and possibly better strategies for
determining accurate antibody saturation conditions.

3.6 Acknowledgements

The authors wish to acknowledge Abe Schwartz and Howard Shapiro for their advice
regarding logarithmic amplification and the linearity of fluorescence intensity values.
We also would like to thank George Stark for kindly providing the transformed
fibrosarcoma cell line and Hoffmann-La Roche for recombinant leukocyte A interferon.
CHAPTER 4

MOBILITY MEASUREMENTS OF IMMUNOMAGNETICALLY LABELED CELLS ALLOW QUANTITATION OF SECONDARY ANTIBODY BINDING AMPLIFICATION

4.1 Introduction

The ability to isolate and analyze cells has important applications in fundamental biological studies as well as in the diagnosis and treatment of human diseases. For example, hematopoietic progenitor cells isolated from human umbilical cord blood, normal bone marrow, and peripheral blood are able to reconstitute hematopoiesis in humans and may be used for the treatment of patients having undergone chemotherapy (de Wynter et al., 1995; Handgretinger et al., 1998). Other medical applications for cell separation include the isolation of putative progenitor cells for angiogenesis (Asahara et al., 1997) and the separation of cancer cells from blood for analysis and early diagnosis (Racila et al., 1998).

Immunological methods such as immunofluorescent, immunomagnetic, and immunomatrix methods are typically employed in order to achieve cell separations and analysis based on the expression of specific molecules on or within the cell. Of these methods, immunomagnetic cell separation is the current interest of our laboratories, more specifically, non-batch, high-throughput quadrupole and dipole immunomagnetic cell
sorting devices (Moore et al., 1998; Sun et al., 1998). The quadrupole magnetic flow sorter has distinct advantages in that it is able to process a larger numbers of cells more quickly than current batch systems. It is also potentially less expensive and orders of magnitude faster than Fluorescent Activated Cell Sorters (FACS). In addition, magnetic flow sorting does not induce the high cellular shear stresses that are present in FACS separation.

Immunomagnetic separation processes commonly employ paramagnetic nanoparticles conjugated to antibodies directed against specific cellular molecules. The use of these paramagnetic nanoparticles, as opposed to paramagnetic microparticles, creates a situation where the volume of magnetic reagent, or degree of immunomagnetic labeling, bound to the targeted cellular molecules is proportional to the expression level of those targeted molecules. Thus, the volume of magnetic reagent bound to a cell will dictate the cell's mobility when that cell is placed in an external magnetic field gradient. Knowledge of this degree of immunomagnetic labeling is a central consideration in the design and operation of continuous immunomagnetic cell separation (Chalmers et al., 1998, 1999a; Moore et al., 1998; Sun et al., 1998, Williams et al., 1999, Zborowski et al., 1999).

The degree of immunomagnetic labeling is obtained using a motion analysis technique to determine the magnetic susceptibility, or magnetophoretic mobility, of an immunomagnetically labeled cell (or particle), on a cell-by-cell basis (Reddy et al., 1998). This technique, also developed in our laboratories, involves videotaping the movement of immunomagnetically labeled cells through a medium of known viscosity and magnetic susceptibility in a well-defined magnetic energy density gradient. The
velocity of each cell along with its location within the magnetic energy gradient is recorded. From this information, the magnetic susceptibility of each cell is calculated. This technique, referred to as Cell Tracking Velocimetry (CTV), includes semi-automated computer video imaging and tracking analysis (Chalmers et al., 1999a, 1999b). A recent advancement in the CTV instrument is in the magnet design, where the magnet now includes a region of nearly constant magnetic energy gradient. Within this specified region, cell mobility is independent of cell position, thus vastly simplifying the data analysis (Chalmers et al., 1999c; Moore et al., 2000).

One of the parameters governing the degree of immunomagnetic labeling and thus, magnetophoretic mobility of an immunomagnetically labeled cell is called the “antibody binding capacity” or $ABC$. $ABC$ is a term that provides quantitative information about the number of antibodies binding to the targeted surface molecules on individual cells. Just as the fluorescent intensity from a Flow Cytometer (FCM) is proportional to cellular $ABC$, it has also been shown that, within a specific range, magnetophoretic mobility is directly proportional to $ABC$ (McCloskey et al., 2000).

In previous papers, a mathematical model and methodology, incorporating CTV technology, were presented which describe using paramagnetically labeled calibration microbeads and a genetically modified fibrosarcoma cell line to correlate magnetophoretic mobility to $ABC$. This correlation was then used to quantitate the $ABC$ of the cells (McCloskey et al., 2000, McCloskey et al., 2001). The mathematical model presented in these papers also includes parameters which indicate that the primary and secondary antibody reagents used in the immunomagnetic labeling procedure influence the final magnetophoretic mobility of an immunomagnetically labeled cell or microbead.
The goal of this manuscript is to show how one may quantitate the antibody binding amplification by comparing measured magnetophoretic mobilities of three different indirect antibody-labeling schemes with the magnetophoretic mobility of a direct antibody-labeling scheme.

4.2 Mathematical Model of Magnetophoresis

For a paramagnetically-labeled cell or microbead, the forces impacting its movement through a liquid suspension are magnetic \( (F_m) \), buoyancy \( (F_{buoy}) \), gravity \( (F_g) \), and drag \( (F_d) \) forces. The magnetic force acting on an immunomagnetically labeled cell or microbead using a two-step labeling protocol has been previously published (McCloskey et al., 2000).

\[
F_m = (n_1 \theta_1 \lambda_1)(n_2 \theta_2 \lambda_2)n_3 F_b \tag{4.1}
\]

\( F_b \) is the magnetic force acting on one paramagnetic nanoparticle in the direction of the magnetic energy gradient. Subscripts "1" and "2" refer to the primary and secondary labeling antibodies, respectively; \( n_1 \) is the number of antigen binding sites per cell, including specific and non-specific antigen sites \( (n_s + n_{ns}) \), and \( \theta_1 \) is the fraction of antigen molecules on the particle surface bound by primary antibody. The parameter, \( \lambda_1 \), represents the valence of the primary antibody binding. For example, it has been shown
that one fully intact anti-CD4 (Leu 3a) antibody typically binds two CD4 cellular antigen molecules, exhibiting classical bivalent antibody binding. In this instance, the value for $\lambda_i$ is $\frac{1}{2}$ (Davis et al., 1998).

The combined term $n_i \theta_i \lambda_i$ is equivalent to the commonly used term “antibody binding capacity” (ABC) of a cell population (Zagursky et al., 1995). Antibody binding capacity is a measure of the number of primary antibodies binding to a cell or microbead. This value includes not only the number of antigen molecules per cell, but also variables such as valence of antibody binding, steric hindrance, binding affinities and non-specific binding. Thus:

$$ABC = n_i \theta_i \lambda_i$$  \hspace{1cm} (4.2)

The same sequence of parameters is then repeated for the binding of the secondary antibody to sites on the primary antibody. In this case, $n_2$ is the number of binding sites on the primary antibody recognized by the secondary antibody. For example, if the secondary antibody is a mouse anti-fluorescein isothiocyanate (FITC), $n_2$ will be the number of FITC molecules conjugated to that primary antibody. This value is often referred to as the fluorescein-to-protein ratio ($F/P$) of the antibody reagent. $\theta_2$ is the fraction of binding sites on the primary antibodies that are bound by secondary antibodies, and $\lambda_2$ represents the valence of the secondary antibody binding (i.e. the number of secondary antibodies that bind per one FITC molecule). These terms $n_2 \theta_2 \lambda_2$ can then be combined into one overall term, $\psi$, representing the antibody amplification
due to the secondary antibody binding to multiple sites on the primary antibody, or the number of secondary antibodies binding per primary antibody.

The final parameter, $n_3$, represents the number of magnetic nanoparticles conjugated to the secondary antibody. (Note that due to a publishing error in McCloskey et al., 2000, the value $\beta_2$ was used in the formulas instead of $n_3$.) Combining parameters $n_2\theta_2\lambda_3 n_3$ into one overall term, $\beta$, gives a value that represents the number of magnetic nanoparticles bound to each primary antibody on a cell or microbead. The lumped term $\beta ABC$ describes the number of magnetic nanoparticles bound to each cell or microbead, and is therefore referred to as the “magnetic particle binding capacity” of a cell or microbead.

The CTV instrument, used to experimentally measure magnetophoretic mobility, is oriented such that the magnetic energy gradient in the experimental system is perpendicular to gravity, thus the governing forces are magnetic force, $F_m$, as described in Equation 4.1, and drag force, $F_d$, which follows Stokes’ law for slow moving particles. Setting these two opposing forces equal to one another, we obtain a relationship for the magnetic energy gradient induced velocity, $v_c$, of the magnetized cell or microbead:

$$v_c = \frac{(n_1\theta_1\lambda_1)(n_2\theta_2\lambda_2)n_3|F_b|}{3\pi D_c\eta} = \frac{ABC \psi n_3|F_b|}{f}$$

where $D_c$ is the diameter of the cell or microbead, $\eta$ is the viscosity of the fluid, and $f$ is the friction coefficient of the moving cell or microbead. Dividing the velocity value by
the magnetophoretic driving force, $S_m$, gives us the magnetophoretic mobility, $m$, a "normalized" parameter analogous to electrophoretic mobility, of the immunomagnetically labeled cell or microbead:

$$m = \frac{\nu_c}{S_m} = \frac{\Delta \chi V_m}{3\pi D_c \eta} n_2 \theta n_3 A B C = \frac{k}{f} \psi n_3 A B C$$

(4.4)

where $\Delta \chi$ is the difference in magnetic susceptibility between the magnetic material, $\chi_b$, and the surrounding medium, $\chi_f$. Also, $V_m$ is the volume of paramagnetic material per paramagnetic nanoparticle. Note that $k = \Delta \chi V_m$ is a constant representing the magnetic property of a single magnetic nanoparticle.

The above equation includes the combined parameters, $n_2 \theta \lambda$, which represent the antibody binding amplification due to the secondary antibody binding to multiple sites on each primary antibody, $\psi$. The work in this manuscript investigates the value of this antibody amplification term. This is accomplished by comparing the magnetophoretic mobilities of two different indirect (two-step) antibody-labeling schemes with the mobility of a direct (single-step) antibody-labeling scheme, all directed against the CD4 antigen on human T-lymphocytes.

4.2.1 Methods for Calculating Secondary Antibody Binding Amplification

The first antibody-labeling scheme uses a mouse anti-CD4 FITC primary antibody and a mouse anti-FITC MACS secondary antibody, Figure 4.1a. The
expression for the magnetophoretic mobility using this first antibody-labeling scheme follows:

\[
m_{1,2f} = \frac{(n_1 \theta_1 \lambda_1)(n_{2f} \theta_{2f} \lambda_{2f})}{f} n_3 k = \frac{(n_1 \theta_1 \lambda_1) \psi_{1,2f} n_3 k}{f} \tag{4.5}
\]

where subscript "1" refers to the mouse anti-CD4 FITC primary antibody and subscript "2f" refers to the mouse anti-FITC MACS secondary antibody. Since the antibody binding amplification factor, \( \psi \), depends on the number of sites on the primary antibody for the secondary antibody to bind, the subscript includes both "1" and "2f" denoting that this factor is dependent on both the primary and secondary antibodies.

The second antibody-labeling scheme incorporates a direct labeling approach using mouse anti-CD4 MACS magnetic nanoparticles as the only antibody, Figure 4.1b. The expression for the magnetophoretic mobility using this second antibody-labeling scheme follows:

\[
m_{1D} = \frac{(n_{1D} \theta_{1D} \lambda_{1D})}{f} n_3 k \tag{4.6}
\]

where subscript "1D" refers to the direct (single-step) mouse anti-CD4 MACS antibody used to label these cells. Here the mouse anti-CD4 MACS antibody was carefully chosen so that it was from the same clone (SK3) as the mouse anti-CD4 FITC-conjugated antibody (used in schemes a, b, and d) and the mouse anti-CD4 unconjugated primary antibody (used in scheme d). Since these products use the same antibody, the only differences between them is that they are unconjugated, conjugated with FITC molecules, or conjugated with magnetic nanoparticles. Assuming that the conjugation of either
FITC molecules or magnetic nanoparticles to the antibody does not affect the antibody’s specificity, valence, or binding affinity, we set \( n_{id} = n_i \), \( \theta_{id} = \theta_i \), and \( \lambda_{id} = \lambda_i \). Now the above equation can be rewritten:

\[
m_{1D} = \frac{(n_i \theta_i \lambda_i) n_3 k}{f}
\]  

Equations 4.5 and 4.7 may now be further manipulated in order to isolate the antibody binding amplification of the secondary antibody, \( \psi \). By dividing Equation 4.7 by Equation 4.5, one obtains:

\[
\frac{m_{1,2f}}{m_{1D}} = \frac{\frac{(n_i \theta_i \lambda_i) n_3 k}{f}}{\frac{(n_i \theta_i \lambda_i) n_3 k}{f}} = \psi_{1,2f}
\]  

where \( \psi_{1,2f} \) represents the antibody binding amplification corresponding to the labeling scheme in Figure 4.1a (the mouse anti-FITC MACS secondary antibodies binding to the FITC sites on the mouse anti-CD4 FITC primary antibody).

The third antibody-labeling scheme uses the same primary mouse anti-CD4 FITC antibody as in the first scheme, but uses a different secondary antibody, a rat anti-mouse MACS antibody, Figure 4.1c. The mathematical expression for the cellular magnetophoretic mobility produced by the third antibody-labeling scheme follows:
where subscript "1" refers to the mouse anti-CD4 FTTC primary antibody and subscript "2m" refers to the rat anti-mouse MACS secondary antibody. Again, by dividing Equation 4.9 by Equation 4.7, one obtains:

$$m_{1,2m} = \frac{(n_1 \theta_1 \lambda_1)(n_{2m} \theta_{2m} \lambda_{2m})n_3 k}{f} = \frac{(n_1 \theta_1 \lambda_1)\psi_{1,2m} n_3 k}{f}$$

(4.9)

where \( \psi_{1,2m} \) represents the antibody binding amplification corresponding to the labeling scheme in Figure 4.1c (the rat anti-mouse MACS secondary antibodies binding to the mouse epitopes on the primary mouse antibody).

The last antibody labeling protocol incorporated a mixture of mouse anti-CD4 FITC-conjugated and unconjugated (pure) primary antibodies and anti-FITC MACS secondary antibodies. This antibody-labeling scheme, depicted in Figure 4.1d, is similar to the first antibody-labeling scheme in Figure 4.1a. The difference between these two schemes is the additional amount of unconjugated antibody that was added to compete with the mouse anti-CD4 FITC-conjugated antibodies. We predicted that this would decrease the
total number of sites available for the secondary antibody to bind. The expression for the magnetophoretic mobility for this fourth antibody-labeling scheme follows:

\[ m_{1,2\varphi} = \frac{(n_1 \theta_1 \lambda_1) (n_{2\varphi} \theta_2 \lambda_{2\varphi}) n_1 k}{f} \]  

(4.11)

where subscript “I” refers to the mouse anti-CD4 FITC-conjugated and mouse anti-CD4 unconjugated primary antibodies and subscript “2\varphi” refers to the number of binding sites specific for secondary antibody binding given that now only a fraction, \( \varphi \), of FITC sites are available as compared with using the anti-CD4 FITC-conjugated antibody alone.

Remembering that \( n_{2f} \) has been defined as the number of binding sites on the primary antibody specific for secondary antibody binding and recognizing that the number of binding sites on the primary antibody for secondary antibody will now be a fraction of the number of sites available as when using mouse anti-CD4 FITC alone, we see that

\[ n_{2\varphi} = \varphi n_{2f} \]  

(4.12)

By dividing Equation 4.11 by Equation 4.5 and substituting in Equation 4.12, we obtain:

\[ \frac{m_{1,2\varphi}}{m_{1,2f}} = \frac{(n_1 \theta_1 \lambda_1) (n_{2\varphi} \theta_2 \lambda_{2\varphi}) n_1 k}{f} = \frac{(n_1 \theta_1 \lambda_1) (n_{2f} \theta_2 \lambda_{2f}) n_1 k}{f} = \varphi \]  

(4.13)
Thus, the decrease in the magnetophoretic mobility due to the addition of the competing antibody is equal to the fraction of the cell molecule antigen sites that are occupied by the FITC conjugated antibody out of the total number of sites occupied by the two antibodies.

**Figure 4.1:** a) CD4 positive lymphocyte cell labeled with an anti-CD4 FITC primary antibody and anti-FITC MACS magnetic nanoparticles (labeling scheme 1) b) CD4 positive lymphocyte cell labeled with an anti-CD4 MACS magnetic nanoparticle (labeling scheme 2) c) CD4 positive lymphocyte cell labeled with a mouse anti-CD4 FITC primary antibody and anti-mouse MACS magnetic nanoparticle (labeling scheme 3) d) CD4 positive lymphocyte cell labeled with FITC conjugated and unconjugated anti-CD4 primary antibodies and anti-FITC MACS magnetic nanoparticles (labeling scheme 4).
4.3 Materials and Methods

4.3.1 Cell Preparation

Whole blood from apparently healthy donors was purchased from the American Red Cross, Central Ohio Region (Columbus, OH). A Ficoll-Paque density gradient centrifugation method (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to obtain the peripheral blood mononuclear (PBMN) cell layer. The excess red blood cells were removed using a lysis buffer consisting of 154 mM NH₄Cl, 10mM KHCO₃, and 0.1 mM EDTA for 10 minutes.

Since monocytes express CD4 molecules as well as T-lymphocytes, the monocytes were removed from the white blood cell population by adhering the monocytes to plastic. The 125 million cells were suspended in 50 ml of RPMI media. Five aliquots of this cell suspension were then poured into T-75 cell culture flasks and incubated for 30 minutes at 37°C allowing the monocytes to adhere to the surface of the flask. The non-adherent cells were collected and washed with a PBS-buffer solution containing 0.1% BSA and 2 mM EDTA in calcium- and magnesium- free phosphate buffer solution (prepared in-house). Aliquots of approximately 4 million enriched PBMNs were placed in four 15 ml centrifuge tubes along with 500 μl of buffer solution and 25 μl of human IgG blocker (Miltenyi Biotec, Auburn, CA). These cells were then immunomagnetically bound by colloidal super-paramagnetic nanoparticles manufactured by Miltenyi Biotec using the four different antibody-labeling schemes described above and in detail below.
4.3.2 Scheme 1: anti-CD4 FITC antibodies and anti-FITC MACS magnetic nanoparticles

A saturating amount, 200 µl (50µl per million cells), of primary monoclonal mouse (Isotype: IgG1, Clone SK3) anti-CD4 (Leu™3a) FITC antibodies (Becton Dickinson Immunocytometry Systems, San Jose, CA, Lot # 15114, F/P = 6.55) was added to the tube and incubated for 30 minutes at 7°C. These cells were then washed once and resuspended in 500 µl of buffer solution. A saturating amount, 200 µl (50µl per million cells), of secondary monoclonal mouse (Isotype: IgG1) anti-FITC MACS antibodies (Miltenyi Biotec, Lot # 5000320036) was then added to the tube and incubated for 30 minutes at 7°C. These cells were then washed twice and resuspended in 2 ml of buffer solution and analyzed by CTV. A diagram of this immunomagnetic labeling scheme is shown in Figure 4.1a. The saturating amounts of antibody reagents were predetermined as described below.

4.3.3 Scheme 2: anti-CD4 MACS magnetic nanoparticles

200 µl of primary monoclonal mouse (Isotype: IgG1, Clone SK3) anti-CD4 (Leu™3a) MACS antibodies (Miltenyi Biotec, Lot # 5000228005) was added to the tube and incubated for 30 minutes at 7°C. These cells were then washed twice and resuspended in 2 ml of buffer solution and analyzed by CTV. A diagram of this immunomagnetic labeling scheme is shown in Figure 4.1b.
4.3.4 Scheme 3: anti-CD4 FITC antibodies and anti-mouse MACS magnetic nanoparticles

A saturating amount, 200 μl, of primary monoclonal mouse (Isotype IgG1, Clone SK3) anti-CD4 (Leu™3a) FITC antibodies (Lot # 15114, F/P = 6.55) was added to the tube and incubated for 30 minutes at 7°C. These cells were then washed once and resuspended in 500 μl of buffer solution and then 200 μl of secondary monoclonal rat anti-mouse IgG1 MACS antibodies (Miltenyi Biotec, Lot # 5990927018) was added to the tube and incubated for 30 minutes at 7°C. These cells were then washed twice and resuspended in 2 ml of buffer solution and analyzed by CTV. A diagram of this immunomagnetic labeling scheme is shown in Figure 4.1c.

4.3.5 Scheme 4: A 50:50 v/v mixture of anti-CD4 antibodies (unconjugated and FITC conjugated) and anti-FITC MACS magnetic nanoparticles

Primary monoclonal mouse (Isotype IgG1, Clone SK3) anti-CD4 (Leu™3a) FITC antibody (Lot # 15114, F/P = 6.55) and of primary monoclonal mouse (Isotype IgG1, Clone SK3) anti-CD4 (Leu™3a) Pure (Becton Dickinson Immunocytometry Systems Lot # 11546, F/P = 0) were both added, 100 μl of each antibody reagent, to the tube and incubated for 30 minutes at 7°C. These cells were then washed once and resuspended in 500 μl of buffer solution. A saturating amount, 200 μl, of secondary monoclonal mouse (Isotype: IgG1) anti-FITC MACS antibodies (Lot # 5000320036) was then added to the tube and incubated for 30 minutes at 7°C. These cells were then washed twice and
resuspended in 2 ml of buffer solution and analyzed by CTV. A diagram of this immunomagnetic labeling scheme is shown in Figure 4.1d.

4.3.6 Antibody Saturation Studies

Experiments were conducted to insure that the concentration of antibody reagent used for cell immunolabeling was sufficient to saturate the available antigen binding sites. To insure this, cells from one blood sample, including monocytes, were incubated with different amounts of the anti-CD4 FITC-conjugated antibody reagent following the protocols above. Figure 4.2 depicts the results from the saturation study for the primary CD4 FITC antibody. Median fluorescence intensities of the CD4 positive lymphocyte cell population from FCM analysis (FACScan, Becton Dickinson Immunocytometry Systems) are plotted versus the volume of CD4 FITC antibody reagent used for 2 million total cells suspended in 250 μl of PBS-buffer solution and 25 μl of human IgG blocker. The 2 million total cells exhibit saturation at 100 μl of antibody reagent.

A separate blood sample was used for the secondary saturation study for the anti-FITC MACS paramagnetic-conjugated antibody. For this study, amounts and volumes described above were scaled-up by a factor of two to accommodate for the larger cell numbers needed for CTV analysis as compared with FCM analysis. Figure 4.3 depicts the mean magnetophoretic mobilities of the CD4 positive lymphocyte cell population from CTV analysis plotted versus the volume of FITC MACS antibody reagent used for 4 million total cells suspended in 500 μl of PBS-buffer solution and 25 μl of human IgG blocker. The optimized amounts, 200 μl of primary CD4 FITC antibody and 200 μl of
secondary MACS antibody per million total cells suspended in 500 µl of PBS-buffer solution and 25 µl of human IgG blocker, were then used for labeling with all reagents in the protocols above.

Figure 4.2: Cellular fluorescence intensity as a function of the added primary anti-CD4 FITC antibody.
**Figure 4.3:** Cellular magnetophoretic mobility as a function of the added secondary anti-FITC MACS antibody.

### 4.3.7 Immunofluorescent Labeling of Quantum Simply Cellular® Microbeads

Quantum Simply Cellular (QSC) calibration microbeads were analyzed using FCM (FACScan, Becton Dickinson Immunocytometry Systems) to check the accuracy of the results from scheme 4 (Figure 4.1d) in which the anti-CD4 FITC-conjugated primary antibodies were mixed with unconjugated anti-CD4 antibodies. Quantum Simply Cellular® microbeads (Flow Cytometry Standards Corporation, San Juan, PR) are polystyrene microbeads of 8 microns in diameter. The microbeads have calibrated
numbers of goat anti-mouse antibodies (GAM) bound to their surfaces. Individual sets of these microbeads are coated with four distinct populations of GAM antibodies that bind the Fc region of IgG1, IgG2a, and IgG2b isotypes of mouse monoclonal antibodies and one blank microbead population for controls. The median ABC values for the population sets are 0, 5,600, 18,000, 51,000, and 150,000.

Aliquots of 100 μl of the QSC microbeads at concentration of 2 million per ml were placed in two different 15 ml centrifuge tubes along with 100 μl of PBS. A saturating amount, 100 μl, of monoclonal mouse (Isotype IgG1, Clone SK3) anti-CD4 (Leu™3a) FITC antibodies (Becton Dickinson Immunocytometry Systems, Lot # 15114, F/P = 6.55) was added to one tube and 50 μl of the same monoclonal mouse anti-CD4 (Leu™3a) FITC antibody along with 50 μl of monoclonal mouse (Isotype IgG1, Clone SK3) anti-CD4 (Leu™3a) Pure (Lot # 11546) were both added to the second tube and incubated for one hour at room temperature. These microbeads were then twice washed with 2 ml of PBS and resuspended in 300 μl of PBS, now ready for FCM analysis.

4.3.8 Cell Concentration Effects on Mobility

In CTV analysis, we make the assumption that there are no cell-cell interactions. If correct, the magnetophoretic mobility of each cell would be independent of the surrounding cells. In order to test for possible evidence of inter-particle interactions, studies have been conducted which measure the concentration effects of well-characterized, magnetite/maghemite-coated polymeric microspheres, approximately 2.7 microns in diameter, mixed with non-magnetic microspheres (microspheres without any
magnetite coating). A complete description and evaluation of these magnetic microspheres has been previously published (Moore et al., 2000).

Four separate microsphere samples were prepared for CTV analysis. The first sample contained a homogeneous population of the non-magnetic microspheres suspended in PBS. The second sample contained a homogeneous population of the magnetite/maghemite-coated magnetic microspheres suspended in PBS. The third sample contained 1 million non-magnetic microspheres mixed with 1 million magnetite-doped microspheres, all suspended in 1 ml of PBS producing a final concentration of 2 million micropheres per ml of PBS. The fourth sample contained 250 thousand non-magnetic microspheres mixed with 250 thousand magnetite-doped microspheres, all suspended in 1 ml of PBS producing a final concentration of 0.5 million micropheres per ml of PBS. The magnetophoretic mobilities of each of these samples were measured by CTV analysis.

4.3.9 The CTV Apparatus

The CTV image analysis system was designed to measure the velocity of the paramagnetically labeled cells or other similar-sized particles in a well-defined magnetic energy gradient. The microbead sample was pumped with a Harvard PhD 2000 Programmable Syringe Pump (Holliston, MA), fitted with a 1 cc syringe, in the z-direction, perpendicular to gravity (x-direction), through flexible tubing into a 1 mm ID square glass channel. An inverted microscope with a 5X objective was focused on the microbeads in the glass channel at the appropriate region of constant force (along the y-direction). Light was supplied to the microscope by a Fiber Lite (Dolan-Jenner,
Lawrence, MA) fiber optic light source with a fiber optic cable. The movement of the cells or microbeads in the magnetic energy gradient (along the z-direction) was videotaped with a 30 Hz Cohu (San Diego, CA) CCD 4915 camera and a Sony SVO-95000MD video recorder. A more thorough discussion of the apparatus can be found in previous publications (Chalmers et al., 1999a, 1999b, 1999c).

4.3.10 CTV Analysis

Analog VCR images from videotapes were converted into 624 X 450 pixel images in which each pixel was assigned a gray level ranging from 0 (black) to 255 (white or brightest) with a μTech image board (Mutech Corp., Billerica, MA) and M-Vision 1000 Sequence Software (Mutech Corp., Billerica, MA). This software allows the user to adjust gain and offset settings to improve image quality before recording and saving images from the videotape or directly from the camera, and converting them into pixel (digital) form. The software also allows the user to choose how many total number of frames, or images, to save as well as how many frames to skip before saving the next image. This frame-skipping feature is an important tool that can sometimes significantly reduce noise in the final velocity data. A more complete discussion of the accuracy of CTV can be found in Nakamura et al. (2001).

Execution of proprietary Borland C++ programs that identify the moving particles and calculate their velocities, allows the velocities for each cell/microbead to be converted into magnetophoretic mobilities using mathematical models. More details are provided in separate publications (Chalmers et al., 1999a, 1999b, 1999c).
4.4 Results

4.4.1 Fluorescent intensities of CD4+ cells

Figure 4.4 is a semi-log histogram depicting the fluorescence intensities of the anti-CD4 labeled PBMNs. This plot illustrates the low level of CD4 expression on monocytes as the “dim” peak, compared with the CD4 positive lymphocyte population, the “bright” peak. The cell population of each peak was verified by gating (side scatter versus forward scatter) for the larger mononuclear cells only, which effectively eliminated the “bright” and the nonfluorescent peak (data not shown). Likewise, gating on the smaller-sized lymphocyte population eliminates the “dim” monocyte peak (data not shown). The results in Figure 4.4 are verified by independent results reporting that monocytes express lower numbers of CD4 antigens, $ABC = 17,000$, than CD4+ T-cells, $ABC = 47,000$ (Bikoue et al., 1996).

4.4.2 Monocyte depletion

Since a heterogeneous population of cells that are expressing multiple levels of CD4 molecules would add variability to the magnetophoretic mobility measurements, the monocyte cell population was depleted from the leukocyte source prior to immunomagnetic labeling. Monocyte depletion was verified using a Coulter Multisizer II instrument for cell size distribution analysis. Figures 4.5 and Figure 4.6 depict the
Figure 4.4: Semi-log histogram showing the fluorescence intensities of peripheral blood mononuclear cells. The brightest cells are the CD4+ T-lymphocytes and the dimly fluorescent cells are the monocytes.
cell size distribution of normal blood cells used in this study before and after monocyte depletion, respectively. The lymphocyte size range fell between 6.1 microns and 8.3 microns while the monocytes size range fell between 8.4 microns and 15 microns. The monocyte depletion process resulted in a reduction of the larger cell peak, from approximately 30% down to 12% of the white blood cell population. The residual red blood cell population also decreased during the monocyte depletion procedure.

![Cell size histogram](image)

**Figure 4.5:** Cell size histogram of the white blood cell population separated on a Ficoll cushion. The largest cells represent the monocyte cell population.
Figure 4.6: Cell size histogram of the white blood cell population separated on a Ficoll cushion after monocyte deletion. Note the decrease in the large cell fraction as compared with panel (a).

4.4.3 IgG blocking

A control study was also conducted on the use of human IgG blocker to block the non-specific binding of the secondary antibody. The linear histograms in Figure 4.7 depict the magnetophoretic mobilities of cells from this control study. The cells were labeled with and without IgG blocking reagent, then non-specifically using secondary
anti-FITC MACS antibodies without any primary antibody. Note that both of these plots are centered approximately at zero velocity, although the mobilities of the non-blocked cells are greater than that of the blocked cells. The mean mobility of the non-blocked cells is \(4.5 \times 10^{-5}\) mm\(^3\)/T-A-s and the mean mobility of the blocked cells is \(-5.2 \times 10^{-6}\) mm\(^3\)/T-A-s (millimeter cubed per tesla-ampere-second). A two-sample t-procedure for comparing means indicated that the IgG blocker is reducing the non-specific binding (\(P < 0.0005\), \(N \sim 400\)).

**Figure 4.7:** Semi-log histogram of the mobilities of the cells labeled with an irrelevant antibody (with and without IgG blocking reagent).
4.4.4 Magnetophoretic mobilities of CD4+ cells

Figures 4.8, 4.10, 4.12, and 4.14 are semi-log histograms depicting magnetophoretic mobility results of the immunomagnetically labeled lymphocytes using the four different antibody-labeling schemes portrayed in Figures 4.1a-d. Figures 4.9, 4.11, 4.13, and 4.15 are the corresponding linear histograms. The distinction between the negative and positively labeled cells is visually observed as the “valley” between the two mobility peaks. Note that for the labeling scheme which produced the highest mobilities as seen in Figures 4.8 and 4.9, the two peaks are clearly separated, unlike the labeling scheme which produced the lowest mobilities as seen in Figures 4.14 and 4.15.

The mobility histograms in Figures 4.8 and 4.9 consist of cells immunomagnetically labeled with mouse anti-CD4 FITC primary antibody, then mouse anti-FITC MACS secondary antibody following antibody labeling scheme 1 as presented in Figure 4.1a. The mean mobility values were calculated using data within the positive peak area only. The mean mobility of the positive peak in Figures 4.8 is \( m_{1,2} = 3.0 \times 10^{-4} \pm 2.2 \times 10^{-5} \) mm\(^3\)/T-A-s (margin of error, 95% confidence limit, N=26).

The cells in Figures 4.10 and 4.11 were immunomagnetically labeled directly with mouse anti-CD4 MACS antibodies following the antibody-labeling scheme 2 presented in Figure 4.1b. The mean mobility of the positive peak in Figures 4.7a is \( m_{1D} = 8.8 \times 10^{-5} \pm 5.5 \times 10^{-6} \) mm\(^3\)/T-A-s (margin of error, 95% confidence limit, N=89).

The cells in Figures 4.12 and 4.13 were immunomagnetically labeled with mouse anti-CD4 FITC primary antibody, then rat anti-mouse MACS secondary antibody following the antibody-labeling scheme 3 presented in Figure 4.1c. The mean mobility
Figure 4.8: Semi-log histogram of the magnetophoretic mobilities (and corresponding velocities) of the cells labeled with anti-CD4 FITC and anti-FITC MACS nanoparticles (labeling scheme 1).
Figure 4.9: Linear histogram of the magnetophoretic mobilities the cells labeled with anti-CD4 FITC and anti-FITC MACS nanoparticles $N_{\text{total}} = 200$. 
Figure 4.10: Semi-log histogram of the magnetophoretic mobilities (and corresponding velocities) of the cells labeled with anti-CD4 MACS nanoparticles (labeling scheme 2).
Figure 4.11: Linear histogram of the magnetophoretic mobilities the cells labeled with anti-CD4 MACS nanoparticles $N_{\text{total}} = 658$. 
Figure 4.12: Semi-log histogram of the magnetophoretic mobilities (and corresponding velocities) of the cells labeled with mouse anti-CD4 FITC and anti-mouse MACS nanoparticles (labeling scheme 3).
Figure 4.13: Linear histogram of the magnetophoretic mobilities the cells labeled with mouse anti-CD4 FITC and anti-mouse MACS nanoparticles $N_{total} = 484$. 
Figure 4.14: Semi-log histogram of the magnetophoretic mobilities (and corresponding velocities) of the cells labeled with FITC conjugated and unconjugated anti-CD4 antibodies and anti-FITC MACS nanoparticles (labeling scheme 4).
Figure 4.15: Linear histogram of the magnetophoretic mobilities the cells labeled with FITC conjugated and unconjugated anti-CD4 antibodies and anti-FITC MACS nanoparticles $N_{total} = 472$. 
of the positive peak in Figures 4.12 is \( m_{1.2m} = 8.6 \times 10^{-5} \pm 4.6 \times 10^{-6} \text{ mm}^3/\text{T-A-s} \) (margin of error, 95% confidence limit, \( N=75 \)).

The cells in Figures 4.14 and 4.15 were immunomagnetically labeled with equal volumes of mouse anti-CD4 FITC primary antibody and of mouse anti-CD4 Pure (unconjugated) primary antibody, then mouse anti-FITC MACS secondary antibody following the antibody-labeling scheme 4 presented in Figure 4.1d. The mean mobility of the positive peak in Figures 4.14 is \( m_{1.2p} = 5.0 \times 10^{-5} \pm 2.7 \times 10^{-6} \text{ mm}^3/\text{T-A-s} \) (margin of error, 95% confidence limit, \( N=154 \)).

4.4.5 Analysis of magnetophoretic mobility amplification

The theory described above was applied to the reported magnetophoretic mobility data in order to calculate the antibody binding amplification, \( \psi_{1.2f} \) and \( \psi_{1.2m} \), resulting from secondary antibodies binding to multiple sites on each primary antibody. By substituting the experimental mobility data from cells labeled according to scheme 1 and scheme 2 into Equation 4.8, we obtained \( \psi_{1.2f} = 3.4 \pm 0.33 \) (margin of error, 95% confidence limit). This indicates that an average of 3.4 anti-FITC MACS secondary antibodies bind to every one primary CD4 FITC antibody. Likewise, from Equation 10 and mobility data from cells labeled according to schemes 2 and 3, we obtained \( \psi_{1.2m} = 0.98 \pm 0.081 \) (margin of error, 95% confidence limit). This indicates that approximately 1 anti-mouse MACS secondary antibody binds to each primary CD4 FITC mouse antibody.
4.4.6 Calculation of the fraction of competing primary antibody

The fourth antibody-labeling scheme incorporated a 50:50 by volume mixture of FITC-conjugated and unconjugated (pure) primary CD4 antibodies. The unconjugated antibody was added to compete with the mouse anti-CD4 FITC-conjugated antibodies. Since fewer FITC conjugated antibodies would be binding to the cells, the total number of sites available for the secondary antibody to bind would also decrease. The proportion of FITC conjugated antibody to unconjugated antibody can be estimated using information provided from the vendor. The provided antibody concentrations were: 3 μg/ml for the FITC-conjugated reagent and 25 μg/ml for the unconjugated CD4 antibodies. Based on this information, and the fact that we used equal volumes of antibody reagent, we estimate that only 11% of the antibodies in the mixture will have the FITC molecules conjugated to them, thus providing binding sites for the secondary antibody.

From Equation 4.13 and mobility data from cells labeled according to the protocol in scheme 4 and scheme 1, we obtained a value for the fraction of FITC sites now available, \( \phi = 0.17 \pm 0.015 \) (margin of error, 95% confidence limit) as compared with the number available in labeling scheme 1. This number indicates that the fraction of binding sites on the primary antibody for secondary antibody binding is only 17% of the total number of sites available compared with when using mouse anti-CD4 FITC alone. This fraction also provides information about the relative concentration of FITC-conjugated and unconjugated (pure) CD4 antibodies in the 50/50 v/v mixture; 17% of the antibodies are FITC-conjugated anti-CD4 antibodies and 83% are unconjugated anti-CD4 antibodies.
The calculated fraction of antibodies in the mixture that have been FITC-conjugated, 17%, is greater than the estimated value of 11% from the vendor information.

The calculated value for $\phi$ may also be obtained using an independent method of analysis, FCM. In FCM analysis, the fluorescence intensity (FI) of an immunofluorescently labeled cell or microbead is proportional to the number of FITC molecules bound to that cell or microbead. Remembering that $n_{2f}$ represents the number of FITC molecules bound to a cell or microbead, we see that the $FI$ of the two immunofluorescently labeled populations may also be used to obtain the fraction of FITC molecules in the antibody mixture:

$$\frac{FI_{2\phi}}{FI_{2f}} = \phi$$

(4.14)

Quantum Simply Cellular calibration microbeads, commonly used in quantitative flow cytometry, were employed to check the validity of the above results. These microbeads were immunofluorescently labeled with the same 50/50 v/v of FITC-conjugated and unconjugated CD4 primary antibodies using the same reagent vials as the reagent vials used to label CD4+ cells following the labeling protocol in scheme 4.

The median fluorescence intensities for each immunofluorescently labeled microbead population were plotted against that microbead’s antibody binding capacity, Figure 4.16. The percentage of FITC-conjugated CD4 antibodies versus unconjugated CD4 antibodies is obtained from a ratio of the median fluorescent intensities of the microbeads. Specifically, the median fluorescence intensity of the microbeads labeled with the
A mixture of FITC-conjugated and unconjugated antibodies is divided by the median fluorescence intensity of microbeads labeled with the FITC-conjugated antibody only. This was done with each for the ABC microbead populations, obtaining five values for $\phi$, and one for the overall linear regressed slope of the two bead suspensions. In remarkable agreement with the results from the mobility measurements from antibody labeling protocol scheme 4 ($\phi = 0.17 \pm 0.015$), these FI measurements from flow cytometry also report an average value of $\phi = 0.17 \pm 0.02$ (standard deviation).

![Figure 4.16](image)

**Figure 4.16:** Linear plot of the median cellular fluorescence intensity versus the corresponding antibody binding capacity of Quantum Simply Cellular calibration microbeads labeled with either FITC conjugated anti-CD4 antibodies and anti-FITC MACS nanoparticles (closed circles), or a mixture of FITC conjugated and unconjugated anti-CD4 antibodies and anti-FITC MACS nanoparticles (open circles).
4.4.7 Magnetophoretic mobilities of magnetic microspheres

In the histograms depicted in Figures 4.8 through 4.15, note that the average mobility of the negative cells is increasing with increasing average mobility of the positive (magnetized) cells. This is most clearly evident by looking closely at the line drawn in each figure separating the two peaks. The separation line is located at \( m = 3 \times 10^{-5} \text{ mm}^3/\text{T-A-s} \) for the least mobile cells in Figure 4.14. This separation line increases to \( m = 5 \times 10^{-5} \text{ mm}^3/\text{T-A-s} \) in Figure 4.10 and Figure 4.12, and increases again to \( m = 1.5 \times 10^{-4} \text{ mm}^3/\text{T-A-s} \) for the most mobile cells in Figure 4.8. This puzzling phenomenon is also consistent with the following observations from CTV analysis.

Mobility measurements of both nonmagnetic and magnetite/maghemite-coated magnetic polymeric microspheres were analyzed on our CTV system. Figure 4.17 displays the mobilities of these two magnetic and nonmagnetic microsphere populations. In this figure, the two populations were analyzed individually as pure populations. Figure 4.18 displays the mobilities of the nonmagnetic microspheres and the magnetic microspheres mixed in the same sample and analyzed together. In comparing Figures 4.17 and 4.18, it is apparent that the mobility of the nonmagnetic microspheres (left peak) increases from a mean mobility of \( m = 2.3 \times 10^{-6} \) to \( m = 1.1 \times 10^{-5} \text{ mm}^3/\text{T-A-s} \) when mixed with magnetic microspheres of higher mobility, while the mobility of the magnetic microspheres (right peak) is not affected by the presence of the nonmagnetic microspheres and remains at a mean mobility of \( m = 1.1 \times 10^{-4} \text{ mm}^3/\text{T-A-s} \). In Figure 4.19, the same sample of mixed magnetic and nonmagnetic beads were analyzed at a
Figure 4.17: Semi-log histogram of the magnetophoretic mobilities of magnetic (right peak) and nonmagnetic (left peak) polymeric microspheres. The microspheres in this plot were analyzed separately as pure populations.
Figure 4.18: Semi-log histogram of the magnetophoretic mobilities of magnetic (right peak) and nonmagnetic (left peak) polymeric microspheres. The microspheres in this plot were mixed together in equal amounts to give a final concentration of 2 million microspheres per milliliter prior to analysis. Note that these peaks are not as well separated as in panel (a).
Figure 4.19: Semi-log histogram of the magnetophoretic mobilities of magnetic (right peak) and nonmagnetic (left peak) polymeric microspheres. The microspheres in this plot were mixed together in equal amounts to give a final concentration of 0.5 million microspheres per milliliter prior to analysis. Note that these peaks are as well separated as in panel (a).
lower concentration 0.5 million beads per milliliter. By comparison, the sample concentration in Figure 4.18 was 2 million beads per milliliter of buffer solution. At the lower microsphere concentration, the mobilities of the nonmagnetic microsphere population do not appear to exhibit a shift towards higher mobilities as the higher concentration sample.

4.5 Discussion

The work in this manuscript investigates the secondary antibody binding amplification, \( \psi \), by comparing two-step antibody labeling protocols with a single antibody labeling protocol. This was accomplished through measuring and comparing the magnetophoretic mobilities of CD4 positive cells, immunomagnetically labeled with a direct and three different indirect protocols. An understanding of these secondary antibody binding interactions is desired since they influence the final magnetophoretic mobility of an immunomagnetically labeled cell, and thus are very important in magnetic cell separation.

It has been shown that an average of 3.4 anti-FITC MACS secondary antibodies bind to each primary CD4 FITC antibody, \( \psi_{1,2} = n_2 \theta_2 \lambda_2 = 3.4 \pm 0.33 \). This result is slightly lower than a previously predicted value for number of anti-FITC MACS secondary antibodies binding to each primary CD2 FITC antibody. The results from this previous study calculated a value for the lumped term \( \beta = n_2 \theta_2 \lambda_2 n_3 = \psi_{\text{calc}} n_3 = 4 \) (McCloskey et al., 2000). Typically, \( n_3 = 1 \) (Kantor et al., 1998), therefore \( \psi_{\text{calc}} = 4 \).
One possible reason for the discrepancy between \( \psi_{1,2f} = 3.4 \) and \( \psi_{\text{calc}} = 4.0 \) is that the magnetic nanoparticles are physically limited from binding to CD4 cells. (Gee et al., 1991; McCloskey et al., 2000) Steric hindrance would act by lowering the number of magnetic nanoparticles that can bind per available binding site, thus lowering the value \( \theta \). It has been calculated that for antibodies exhibiting this level of amplification, steric hindrance effects will begin above approximately 30,000 ABC (McCloskey et al., 2000). Since it has also been well documented that T-cell associated CD4 molecules exhibit approximately 47,000 ABC (Bikoue et al., 1996; Davis et al., 1998; Poncelet, 1999), we would expect to see some amount of steric hindrance on the CD4 immunomagnetically labeled cells. We conclude that the lower value of \( \psi_{1,2f} = 3.4 \) compared with \( \psi_{\text{calc}} = 4.0 \) for the immunomagnetically labeled CD4 positive cells is due to steric hindrance limiting the binding of the magnetic nanoparticles to the cellular surface.

For the cells labeled according to the protocol in scheme 3, the secondary antibody binding amplification was calculated to be \( \psi_{1,2m} = 0.98 \pm 0.081 \). In other words, there was approximately one anti-mouse MACS secondary antibody binding to each primary CD4 FITC mouse antibody on the CD4 positive lymphocytes. At this low level of antibody binding, the steric hindrance described above would be negligible. The current method does not allow one to distinguish between possible secondary antibody binding mechanisms. One possible binding mechanism involves only one IgG1 mouse site per anti-mouse MACS antibody with monovalent antibody binding, in which case \( n_{2m} = 1 \), \( \theta_{2m} = 0.98 \), and \( \lambda_{2m} = 1 \). The second binding mechanism involves two mouse sites per
anti-mouse MACS antibody with bivalent antibody binding, meaning that each rat anti-
mouse antibody binds two mouse antigen sites on each primary antibody, in which case

\[ n_{2m} = 2, \theta_{2m} = 0.98, \text{ and } \lambda_{2m} = 1/2. \]

From the magnetophoretic mobility data in this study (Figures 4.8-4.15), it appears as if the mobile cells are imparting a very small mobility to the nonmobile cells and that this mobility is proportional to the mobility of the mobile cells. In addition, the magnetophoretic mobility results using magnetic microspheres depicted in Figures 11a-c verifies this phenomenon and indicates that this effect on the nonmobile microspheres is concentration dependent. At lower concentrations, the mobilities of the microsphere populations do not appear to exhibit the same shift towards higher mobilities as the higher concentration sample. Our labs are continuing to investigate the cause of these shifting mobilities of the nonmagnetic cell and microsphere populations.

The results from Figures 4.17, 4.18 and 4.19 using magnetite-doped polymer microspheres, are important in analysis of CD4 cellular mobilities because these results immediately eliminates two other factors as possibly responsible for the increase of the negative cell population with increasing mobility of the CD4 positive cell population. These two other factors include non-specific antibody binding or monocyte contamination. Also, since this observable phenomena of shifting mobilities does not appear to affect the positive, faster moving cell population, we are confident in our mobility calculations for each of the antibody-labeling schemes presented in this work.

The overall goal of our research is to develop magnetic separation devices and techniques. The ability to impart magnetophoretic mobility on a target cell is a crucial part of any magnetic separation technique. Mathematical equations have been proposed
which model the magnetic nanoparticle antibody binding mechanisms used to impart this magnetophoretic mobility on a target cell (McCloskey et al., 2000). In addition, the primary antibody binding parameters, $n_1 \theta_1 \lambda_1$, or antibody binding capacity (ABC), have been investigated and quantitated (McCloskey et al., 2000; McCloskey et al., 2001). The work presented in this manuscript continues examination of the antibody binding mechanisms to study the amplification of magnetophoretic mobility due to secondary antibody binding parameters, $\psi = n_2 \theta_2 \lambda_2$. Combined, these three publications provide a significant amount of information about the antibody binding mechanisms involved in immunomagnetically labeling a target cell for magnetic separation.

4.6 Acknowledgments

This work has been supported by grants from the National Science Foundation (BCS-9258004, BES-9731059 to J.J.C.) and the National Cancer Institute (R01 CA62349 to M.Z. and R33 CA81662-01 to J.J.C.).
CHAPTER 5

CHARACTERIZATION OF MAGNETOPHORETIC MOBILITY:
AN OVERVIEW

5.1 Introduction

The magnetophoretic mobility of an immunomagnetically labeled cell (Figure 5.1) is an important factor in continuous magnetic cell separation. It has been shown that several factors contribute to an immunomagnetically labeled cell’s magnetophoretic mobility. (McCloskey et al., 2000, 2001a, 2001b) This paper combines the information learned from this previous work and then extends the data to further investigate and estimate the contributions of each parameter controlling magnetophoretic mobility and the range of magnetophoretic mobilities that can be expected under different magnetic labeling conditions.

5.2 Theory of Magnetophoretic Mobility

As published previously, the overall equation for magnetophoretic mobility is:
Figure 5.1: Diagram of an immunomagnetically labeled cell.

Subscripts "1" and "2" refer to the primary and secondary labeling antibodies, respectively; \( n_l \) is the number of antigen binding sites per cell, including specific and non-specific antigen sites \((n_s + n_{ns})\), and \( \theta_l \) is the fraction of antigen molecules on the particle surface bound by primary antibody. The parameter, \( \lambda_l \), represents the valence of the primary antibody binding. The combined term \( n_l \theta_l \lambda_l \) is equivalent to the commonly
used term “antibody binding capacity” (ABC) of a cell population (Zagursky et al., 1995). Antibody binding capacity is a measure of the number of primary antibodies binding to a cell or microbead. This value includes not only the number of antigen molecules per cell, but also variables such as valence of antibody binding, steric hindrance, binding affinities and non-specific binding.

The same sequence of parameters is then repeated for the binding of the secondary antibody to sites on the primary antibody. In this case, \( n_2 \) is the number of binding sites on the primary antibody recognized by the secondary antibody. \( \theta_2 \) is the fraction of binding sites on the primary antibodies that are bound by secondary antibodies, and \( \lambda_2 \) represents the valence of the secondary antibody binding. These terms \( n_2 \theta_2 \lambda_2 \) can then be combined into one overall term, \( \psi \), representing the antibody amplification due to the secondary antibody binding to multiple sites on the primary antibody, or the number of secondary antibodies binding per primary antibody.

The final parameter, \( n_3 \), represents the number of magnetic nanoparticles conjugated to the secondary antibody. (Note that due to a publishing error in McCloskey et al., 2000, the value \( \beta_2 \) was used in the formulas instead of \( n_3 \).) Combining parameters \( ABC \psi n_3 \) into one overall term, \( N_{np} \), gives a value that represents the number of magnetic nanoparticles bound to each cell or microbead, and is therefore referred to as the “magnetic particle binding capacity” of a cell or microbead.

Other parameters include the friction coefficient of the moving cell or microbead, \( f = 3\pi D_c \eta \), where \( D_c \) is the diameter of the cell or microbead and \( \eta \) is the viscosity of the fluid. \( \Delta \chi \) is the difference in magnetic susceptibility between the magnetic material, \( \chi_b \), and the surrounding medium, \( \chi_f \), and \( V_m \) is the volume of paramagnetic material per
paramagnetic nanoparticle. Note that $\Delta \chi V_m$ is a constant representing the magnetic property of a single magnetic nanoparticle.

By combining terms and simplifying Equation 5.1 for the magnetophoretic mobility of an immunomagnetically labeled cell, we have:

$$m = \frac{ABC \psi n_3 \Delta \chi V_m}{3\pi D_c \eta} = \frac{N_{mp} \Delta \chi V_m}{3\pi D_c \eta}$$

(5.2)

Here we see that the parameters that influence magnetophoretic mobility include:

$$m = f(ABC, \psi, n_3, \Delta \chi V_m, D_c, \eta)$$

(5.3)

In CTV experiments, the viscosity of the fluid is controlled and then number of magnetic particles conjugated the specifically binding antibody is usually 1 (Kantor et al., 1998), therefore, the most influential parameters on magnetophoretic mobility are $ABC$, $\psi$, $D_c$, and $\Delta \chi V_m$. The $ABC$ of a cell population depends on cell biology and the antibody binding mechanism (i.e. monovalent or bivalent antibody binding) of the antibody used. Commonly, a cell’s $ABC$ can range from 1 to 1 million. $\psi$ usually ranges from 1 to 5 and is more limited due to steric hindrance of the magnetic particles. $D_c$ commonly ranges from 5 to 20 µm, but within a cell subset population $D_c$ will vary by only a few microns. $\Delta \chi V_m$ has the potential for significant variability depending on the size and composition of the magnetic particles. This paper will investigate the influentialalbility of each of these parameters on magnetophoretic mobility: $ABC$, $\psi$, $D_c$, and $\Delta \chi V_m$. First, will be the
examination of the parameter $\Delta \chi V_m$ for two different magnetic nanoparticles over a range of $N_{mp}$.

5.3 $\Delta \chi V_m$

The value of $\Delta \chi V_m$ is dependent on the choice of magnetic particles that are conjugated to the antibody. This value includes the magnetic particle size (depicted in Figure 5.2) and the magnetic susceptibility of the magnetic material in the magnetic particle. The magnetic susceptibility can range from diamagnetic, like water, to superparamagnetic (Table 5.1). The magnetic particles used in most of our previous studies are ironoxide compounds, which are considered ferromagnetic compounds rather than paramagnetic or diamagnetic. The magnetic susceptibility of ferromagnetic materials is more complicated because the magnetic susceptibility of ferromagnetic materials is dependent on the magnetic field strength, whereas the magnetic susceptibility of paramagnetic and diamagnetic compounds are independent of magnetic field strength.

The data in Figure 5.3 compares the effect of $\Delta \chi V_m$ on mobility for two different ironoxide ferromagnetic nanoparticles; MACS from Miltenyi Biotech and Immunicon nanoparticles. The value for the magnetic potency of MACS nanoparticles have been previously experimentally derived, $\Delta \chi V_m = 2.5 \times 10^{-16} \text{ mm}^3$. (McCloskey et al. 2000) Using this experimentally calculated $\Delta \chi V_m$ value for MACS nanoparticles, an estimated value of $\Delta \chi V_m$ can be calculated for the slightly larger Immunicon nanoparticles mean diameters: 140nm for Immunicon and 50nm for MACS nanoparticles and w/w %
magnetic material compositions of: 80% for Immicon and 55% for MACS. This estimation of the $\Delta \chi V_m$ for Immicon assumes that both Immicon and MACS paramagnetic nanoparticles are composed of identical paramagnetic materials. Note that for equal numbers of magnetic nanoparticles binding to a cell, the mobilities for the Immicon magnetic nanoparticles are almost two orders of magnitude greater than the mobilities for the smaller, less magnetic MACS nanoparticles.

**Figure 5.2**: Diagram of cells immunomagnetically labeled with two different sized magnetic particles.
<table>
<thead>
<tr>
<th>Substance</th>
<th>Formula</th>
<th>Temperature K</th>
<th>Susceptibility $10^{-6}$ cgs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysprosium</td>
<td>Dy</td>
<td>293.2</td>
<td>103,500</td>
</tr>
<tr>
<td>Erbium</td>
<td>Er</td>
<td>291</td>
<td>44,300</td>
</tr>
<tr>
<td>Gadolinium</td>
<td>Gd</td>
<td>300.6</td>
<td>755,000</td>
</tr>
<tr>
<td>Homium</td>
<td>Ho</td>
<td>293</td>
<td>88,100</td>
</tr>
<tr>
<td>Iron</td>
<td>Fe</td>
<td>........</td>
<td>ferro</td>
</tr>
<tr>
<td>IronOxide</td>
<td>FeO</td>
<td>293</td>
<td>7,200</td>
</tr>
<tr>
<td>IronOxide</td>
<td>Fe$_2$O$_3$</td>
<td>1033</td>
<td>3,586</td>
</tr>
<tr>
<td>IronOxide</td>
<td>Fe$_3$O$_4$</td>
<td>........</td>
<td>ferro</td>
</tr>
<tr>
<td>Terbium</td>
<td>Tb</td>
<td>273</td>
<td>146,000</td>
</tr>
<tr>
<td>Water</td>
<td>H$_2$O</td>
<td>293</td>
<td>-13</td>
</tr>
</tbody>
</table>

**Table 5.1:** Table listing the magnet susceptibilities of paramagnetic and diamagnetic elements and inorganic compounds. (CRC Handbook, 1980)
Figure 5.3: Logarithmic plot of the magnetophoretic mobilities over a range of the number of magnetic particles bound to an immunomagnetically labeled cell or microbead for two different magnetic particles: MACS and Immunicon.

5.4 Antibody Binding Capacity

In addition to the size and magnetic potency of the magnetic particles ($\Delta \chi V_m$), the antibody binding capacity is another important factor affecting the magnetophoretic mobility of an immunomagnetically labeled cell. As seen in Figure 5.4 and 5.5, increasing the antibody binding capacity will increase the number of magnetic particles binding to a cell, thus increase the magnetophoretic mobility of a cell as well. It has been experimentally shown that ABC is linearly proportional to magnetophoretic mobility within a limited region. (Figure 5.5)
Figure 5.4: Diagram of immunomagnetically labeled cells with different ABC values.
Figure 5.5: Linear plot of magnetophoretic mobility versus the ABC of QSC calibration microbeads. This plot is also presented in Chapter 2 and 3.

It has also been calculated that this limited region is due steric hindrance of the magnetic particle binding and is therefore dependent on the cell size and size of the magnetic particles. (McCloskey et al., 2001) The size of current commercially available immunomagnetic particles ranges from 10nm to 7 microns. Based on this calculation of steric hindrance, the maximum number of magnetic particles can be calculated for the size range of immunomagnetic particles. Figure 5.6 is a plot of the number of magnetic particles that can bind to a cell 7 microns in diameter.
Figure 5.6: Logarithmic plot of the maximum number of magnetic particles which can bind to a cell (diameter = 7 \( \mu \)m) versus the size of the magnetic particles.

In the above mathematical model, the ABC is assumed to be independent of the size of an individual cell. Assuming that these two parameters are unrelated, there are two possible scenarios of magnetic particle binding. The first is that the binding of large magnetic particles will limit by the space available for these magnetic particles to bind. In this instance, the ABC becomes less important and the mobilities of the cells are expected to be entirely dependent on the variation in the size and potentancy of the large
magnetic particles. The second scenario is that for small magnetic particles, which will not exhibited limited binding and, therefore, \( N_{mp} \) will be linearly related to \( ABC \) as in the linear region in Figure 5.5.

However, in some specific cases, the expression on antigens on a cell's surface may be related to the surface area on the cell. Assuming this to be true, the number of binding sites would be proportional to the size, \( D_o \), of the cells. For these situations, a constant value, \( \alpha \), equal to \( n_i \) divided by the surface area of the cell, \( S_{Ac} \), can be incorporated into the mathematical model.

\[
\alpha = \frac{n_i}{S_{Ac}}
\]  

(5.4)

5.5 Secondary Antibody Binding Amplification, \( \psi \)

In characterization of the paramagnetic labeling, one of the parameters of interest is the increase in magnetophoretic mobility due to secondary antibody binding to multiple epitopes (Figure 5.7) on the primary antibody, referred to as the “secondary antibody binding amplification,” \( \psi \). Secondary antibody binding amplification has been investigated and quantitated by comparing the mobilities from lymphocytes directly labeled with anti-CD4 MACS magnetic nanoparticles with the mobilities of the same lymphocytes labeled with two different indirect antibody-labeling schemes. Each indirect labeling scheme incorporated a primary mouse anti-CD4 FITC antibody and either of two different secondary antibodies: anti-FITC MACS magnetic nanoparticles or
**Figure 5.7**: Diagram of cells immunomagnetically labeled with a direct and indirect antibody labeling scheme. Notice that the primary antibody in the indirect antibody labeling scheme provides additional binding sites for the secondary antibody, thus we have an amplification in the number of secondary antibodies binding and, therefore, in the number of magnetic particles binding as well.

Anti-mouse MACS magnetic nanoparticles. Results indicate that an average of 3.4 anti-FITC MACS secondary antibodies bind to each primary CD4 FITC antibody, $\psi_{1.3} = 3.4 \pm 0.33$, and that approximately one, $\psi_{1.2m} = 0.98 \pm 0.081$, anti-mouse MACS secondary antibody binds to each primary mouse CD4 FITC antibody on a CD4 positive lymphocyte. (McCloskey et al., 2001) Figure 5.8 is a theoretical plot of the number of magnetic particles that bind versus the ABC of the cell population. Notice that the slope of each line is a function of the antibody amplification.
Figure 5.8: Linear plot of the number of magnetic particles binding to a cell versus the antibody binding capacity of that cell. The slope of this linear relationship is proportional to the secondary antibody binding amplification, $\psi$.

5. 6 Variables contributing to large distribution in $m$

Figure 5.9 shows the typical distribution in magnetophoretic mobilities for an immunomagnetically labeled cell population. As typical for biological systems, the measured magnetophoretic mobilities exhibit large distributions. The factors contributing to this large distribution include the inherent distribution in cell sizes, $D_c$, the inherent distribution in the number of antigen molecules expressed on a cell, $ABC$, the range in
secondary antibody binding amplification, \( \psi \), and the distribution in the size and composition, \( \Delta \chi V_m \), in the magnetic particles bound to the cell.

Figure 5.9: Logarithmic plot of the magnetophoretic mobilities of a typical immunomagnetically labeled cell population.
With some information about a cell population's size distribution and ABC range, it is possible to make some estimates as to the relative contributions of each of the listed parameters in the distribution in magnetophoretic mobility. The variation in the size of a cell population will commonly range only a few microns. Assuming a mean diameter of 7μm ± 1μm, the effect of the cell's diameter on the magnetophoretic mobility can be calculated. This calculation assumes that the antigen expression on a cell is independent of the cell diameter, that small magnetic particles, so steric hindrance will not be a contributing factor, and that the only affect on mobility due to the size of a cell is from the drag force on a cell, which will increase for larger cells. The mean ABC and standard deviation of a CD4 lymphocyte has been calculated to be $ABC = 47,000 ± 14,000$ (Bikoue et al., 1996) The mean value for the secondary antibody binding amplification, $\psi$, for anti-FITC MACS nanoparticles has been calculated to be $3.4 ± 1$ (McCloskey et al., 2000b) The mean size of MACS nanoparticles is $50 ± 30$ nm consisting of 55-59% magnetitie/maghemite material.

For a large number of magnetic particles binding per cell, as in the very small MACS nanoparticles, the variation in the value and will be averaged over several thousand binding sites. For this reason, the variation in the mobility from the variation in the parameters $\psi$ and $\Delta \chi V_m$ will have a negligible effect from cell to cell. Therefore, the two most influential parameters contributing to the variation in mobility are $ABC$ and $D_c$. Based on the above values for the mean and standard deviation of $ABC$ and $D_c$, one can calculate the percentage of each variable contributing to the variation in mobility. These results are seen in Figure 5.10.
Figure 5.10: Plot of the relative contributions of the two factors which contribute to the large variation in magnetophoretic mobilities.
5.7 Conclusions

The factors governing magnetophoretic mobility include such parameters as the biological state of the cell: the cell’s size and its antigen expression level, the specific antibodies and antibody labeling scheme, and the magnetic particles chosen for antibody conjugation. These parameters are also related to and influence one another as well. The work in this chapter has attempted to investigate the range of these parameters, their scope, and limitations as they relate to the number of magnetic particles that can bind to a cell and the immunomagnetically labeled cell’s magnetophoretic mobility.
CHAPTER 6

ANTIBODY BINDING CAPACITY AND MAGNETIC CELL SEPARATION

6.1 Introduction

Antibody binding capacity (ABC) is a term representing a cell's ability to bind antibodies, often correlating with the number of specific cellular antigens expressed on that cell. ABC allows paramagnetic-conjugated antibodies to impart magnetophoretic mobility on the targeted cells, which enables the user to separate the specifically tagged mobile cells from the non-mobile cells. The cell's ABC distribution and sample-to-sample variation is a complicating factor in continuous magnetic cell separation. This work investigates the relationship between ABC and magnetic separation efficiency by injecting a suspension of immunomagnetically-labeled Quantum Simply Cellular calibration microbeads into fluid flowing through a quadrupole magnetic sorter and evaluating the elution profiles of the outlet streams using UV detectors. Ideal separation flow rates were shown to be a function of ABC, and thus a function of the
magnetophoretic mobilities. Comparing experimental and theoretical results, the theory correctly predicted maximum separation flow rates, but overestimated the separation purities.

6.2 Mathematical Model of Magnetophoresis

The magnetic force acting on an immunomagnetically labeled cell or microbead using a two-step labeling protocol has been previously published (McCloskey et al., 2000).

\[
F_m = (n_1 \theta_1 \lambda_1)(n_2 \theta_2 \lambda_2)n_z F_b
\]  

(6.1)

\(F_b\) is the magnetic force acting on one paramagnetic nanoparticle in the direction of the magnetic energy gradient. Subscripts “1” and “2” refer to the primary and secondary labeling antibodies, respectively; \(n_z\) is the number of antigen binding sites per cell, including specific and non-specific antigen sites \((n_s + n_{ns})\), and \(\theta_1\) is the fraction of antigen molecules on the particle surface bound by primary antibody. The parameter, \(\lambda_1\), represents the valence of the primary antibody binding. For example, it has been shown that one fully intact anti-CD4 (Leu 3a) antibody typically binds two CD4 cellular antigen molecules, exhibiting classical bivalent antibody binding. In this instance, the value for \(\lambda_1\) is \(\frac{1}{2}\) (Davis et al., 1998).

The combined term \(n_z \theta_1 \lambda_1\) is equivalent to the commonly used term “antibody binding capacity” \((ABC)\) of a cell population (Zagursky et al., 1995). Antibody binding capacity is a measure of the number of primary antibodies binding to a cell or microbead.
This value includes not only the number of antigen molecules per cell, but also variables such as valence of antibody binding, steric hindrance, binding affinities and non-specific binding. Thus:

$$ABC = n_1 \theta_1 \lambda_1$$  \hspace{1cm} (6.2)

The same sequence of parameters is then repeated for the binding of the secondary antibody to sites on the primary antibody. In this case, $n_2$ is the number of binding sites on the primary antibody recognized by the secondary antibody. For example, if the secondary antibody is a mouse anti-fluorescein isothiocyanate (FITC), $n_2$ will be the number of FITC molecules conjugated to that primary antibody. This value is often referred to as the fluorescein-to-protein ratio ($F/P$) of the antibody reagent. $\theta_2$ is the fraction of binding sites on the primary antibodies that are bound by secondary antibodies, and $\lambda_2$ represents the valence of the secondary antibody binding (i.e. the number of secondary antibodies that bind per one FITC molecule). These terms $n_2 \theta_2 \lambda_2$ can then be combined into one overall term, $\psi$, representing the antibody amplification due to the secondary antibody binding to multiple sites on the primary antibody, or the number of secondary antibodies binding per primary antibody.

The final parameter, $n_3$, represents the number of magnetic nanoparticles conjugated to the secondary antibody. (Note that due to a publishing error in McCloskey et al., 2000, the value $\beta_2$ was used in the formulas instead of $n_3$.) Combining parameters $n_2 \theta_2 \lambda_2 n_3$ into one overall term, $\beta$, gives a value that represents the number of magnetic nanoparticles bound to each primary antibody on a cell or microbead. The lumped term
\( \beta ABC \) describes the number of magnetic nanoparticles bound to each cell or microbead, and is therefore referred to as the "magnetic particle binding capacity" of a cell or microbead. The final equation, as previously published (McCloskey et al., 2000) for the magnetophoretic mobility of an immunomagnetically labeled cell is:

\[
m = \frac{\Delta \chi V_m}{3\pi D_c \eta} n_2 \theta_2 \lambda_2 n_3 ABC = \frac{k}{f} \psi n_3 ABC
\]

where \( D_c \) is the diameter of the cell or microbead, \( \eta \) is the viscosity of the fluid, and \( f \) is the friction coefficient of the moving cell or microbead, \( \Delta \chi \) is the difference in magnetic susceptibility between the magnetic material, \( \chi_b \), and the surrounding medium, \( \chi_f \). Also, \( V_m \) is the volume of paramagnetic material per paramagnetic nanoparticle. Note that \( k = \Delta \chi V_m \) is a constant representing the magnetic property of a single magnetic nanoparticle.

6.3 Materials and Methods

6.3.1 Quantum Simply Cellular® Microbeads

Quantum Simply Cellular® microbeads (Flow Cytometry Standards, Corp., San Juan, PR) are uniform, 8.5 micron polystyrene microbeads with calibrated numbers of goat anti-mouse antibodies (GAM) bound to their surfaces. Individual sets of these microbeads are coated with four distinct populations of GAM antibodies that bind the Fc region of IgGl, IgG2a, and IgG2b isotypes of mouse monoclonal antibodies and one
blank microbead population for controls. Quantum Simply Cellular® microbeads were custom -shipped in individually packaged 5 vials of uniform antibody binding populations having median ABC values ranging from approximately 0 to 210,000. Each vial of these microbeads contains 2 million microbeads per ml.

6.3.2 Antibody Saturation Studies

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

6.3.3 Labeling QSC Microbeads for CTV Analysis

Approximately 1.5 million (750 µl) of the QSC microbeads (Lot # A-03366) were placed in centrifuge tubes and adjusted to 1.5 million microbeads per ml with PBS. Saturating amounts (15.5 µl) of primary mouse (Isotype IgG2a) anti-CD2 FITC antibodies (CD2 Leu™5b, Becton Dickinson Immunocytometry Systems, San Jose, CA,
Lot # 00199) were added to the tubes and incubated for one hour at 7°C. These microbeads were then washed with PBS. The immunofluorescently labeled microbeads were resuspended in 500 μl of PBS and then given saturating amounts (50 μl) of secondary mouse (Isotype IgG1) MACS anti-FTTC antibodies (Miltenyi Biotec, Auburn, CA, Lot # NE8163) and incubated for 30 minutes at 7°C. These microbeads were then washed twice with PBS and resuspended in 750 μl of PBS for CTV analysis.

6.3.4 The CTV Apparatus

The CTV image analysis system was designed to measure the velocity of the paramagnetically labeled cells or other similarly sized particles in a well-defined magnetic energy gradient, depicted in Figure 2.3. The sample was pumped with a Harvard PhD 2000 Programmable Syringe Pump (Holliston, MA), fitted with a 1 cc syringe, in the negative z-direction through flexible tubing into a 1 mm ID square glass channel. An inverted microscope with a 5X microscope objective was focused on the microbeads in the glass channel at the appropriate region of constant force. Light was supplied to the microscope by a Fiber Lite (Dolan-Jenner, Lawrence, MA) fiber optic light source with a fiber optic cable. The movement of the cells or microbeads in the magnetic energy gradient was videotaped with a 30 Hz Cohu (San Diego, CA) CCD 4915 camera and a Sony SVO-95000MD video recorder. A more thorough discussion of the apparatus can be found in previous publications. (Chalmers et al., 1999a, 1999b, and 1999c, Nakamura et al., 2000)
6.3.5 CTV Analysis

Analog VCR images from videotapes were converted into 624 X 450 pixel images in which each pixel was assigned a gray-level ranging from 0 (black) to 255 (white or brightest) with a µTech image board (Mutech Corp., Billerica, MA) and M-Vision 1000 Sequence Software (Mutech Corp., Billerica, MA). This software allows the user to adjust gain and offset settings to improve image quality before recording and saving images from the videotape or directly from the camera and converting them into pixel (digital) form. The software also allows the user to choose how many total frames of images to save as well as how many frames to skip before saving the next image. This frame-skipping feature is an important tool that can sometimes significantly reduce noise in the final velocity data. (Nakamura et al., 2000)

Execution of proprietary Borland C++ programs that identify the moving particles and calculate their velocities allow the velocities for each cell/microbead tracked to be converted into magnetophoretic mobilities using mathematical models. More details are provided in separate publications. (Chalmers et al., 1999a, 1999b, 1999c)

6.3.6 Labeling QSC Microbeads for Pulse-Injection into the Quadrapole Magnet

Approximately 4 million (2 ml) of the QSC microbeads (Lot # A-072199) were placed in centrifuge tubes and adjusted to 1.5 million microbeads per ml with PBS. Saturating amounts (55 µl) of primary mouse (Isotype IgG2a) anti-CD2 FITC antibodies
(CD2 Leu-™5b, Becton Dickinson Immunocytometry Systems, San Jose, CA, Lot #12609 or 70549) were added to the tubes and incubated for one hour at 7°C. These microbeads were then washed with PBS. The immunofluorescently labeled microbeads were resuspended in 1.4 ml of PBS and then given saturating amounts (200 µl) of secondary mouse (Isotype IgG1) MACS anti-FITC antibodies (Miltenyi Biotec, Auburn, CA, Lot #NE9814) and incubated for 30 minutes at 7°C. These microbeads were then washed twice with PBS and resuspended in 2 ml of PBS. The final concentration was approximately 1.5 million per ml.

6.3.7 Pulse Injection into Quadrupole Magnetic Flow Sorter

The elution of the magnetically labeled particles was conducted using a quadrupole magnetic flow sorting device as previously described in Sun et al., 1998 and a pulse injection techniques as described in Hoyos et al., 2000. A diagram of the experimental set-up is presented in Figure 6.1. The QMS channel is composed of an inner rod and an outer cylinder. Splitters are placed at the inlets and outlets to physically separate the inlet and outlet streams. At the center of the channel, the particles are exposed to a magnetic energy gradient that pulls magnet particles outward into the b stream. The flow-rates, Q, of inlet streams a' and b' and outlet stream a were controlled by two multi-syringe pumps (Type 33 syringe pump; Harvard Instruments, Natick, MA, USA) The outlet stream b was left open to the atmosphere. The particles sample was injected into inlet stream a' with a six-way valve and 50 µl injection volume loop. (Rheodyne 7725i; Alltech
Figure 6.1: Diagram of experimental set-up for pulse-injecting samples into a quadrupole continuous magnetic cell sorter.
Associates, Deerfield, IL, USA) Two online UV detectors with 32-μl flow cells (VUV-10; HyperQuan, Colorado Springs, CO, USA) were attached to the $a$ and $b$ outlet streams of the QMS separator to measure the absorbance of the particles eluting from the two streams. The detector signal was fed into an analog-to-digital converter (DI-190, Dataq Instruments, Akron, OH, USA) and stored in an IBM-compatible personal computer. The data were analyzed using appropriate software for peak deconvolution (WinDaq Lite, Dataq Instruments and PeakFit, SPSS, Chicago, IL, USA).

As seen in Figure 6.2, the magnetically labeled particles injected into the inner stream $a'$ will travel outward towards the $b$ streams as they are carried down the channel. If the volumetric flow rate, $Q$, is slow enough, the particles will have enough time to move into and elute in the $b$ stream. Alternatively, if the volumetric flow-rate is too fast, then the particles will not have enough time to travel into the $b$ stream and will elute in the $a$ stream. In addition, if the volumetric flow-rate is very slow, then the particles might strike the wall and be retained along the walls in the column. These experiments were conducted by injecting samples of immunomagnetically labeled particles into the QMS sorter and varying the total volumetric flow-rates. The inlet and outlet flow rate ratios, $Q_{a}/Q$ and $Q_{a}/Q$, were kept constant at 0.1 and 0.25 throughout all of the experiments. The flow conditions giving the maximum recovery in $F_{b}$ was evaluated for each QSC microbead population and these ideal flow conditions were then correlated with the ABC values for each QSC microbead population.
Figure 6.2: Schematic showing the separation of magnetic particles into b stream
6.3.8 Calibration of elution curve

The absorbance, $A$, from the elution peaks in the $a$ and $b$ outlet streams correlate with the number or total mass of particles which pass through the UV detectors.

$$A = kc$$  \hspace{1cm} (6.4)

where $A$ is the absorbance, $k$ is a constant dependent on the particle size and material, and $c$ is the concentration of particles in suspension:

$$c = \frac{n}{V}$$  \hspace{1cm} (6.5)

$n$ is the number of particles and $V$ is the volume of the liquid suspension.

As the particles flow through the UV detectors at a constant time, $t$, the absorbance, $A$, increases to a maxima and then decreases down to the baseline as the particles elute from the detector. The area, $h$, under this elution curve for detector $b$ is represented as:

$$h_b = \sum A(t) \Delta t = k \sum c(t) \Delta t = \frac{k}{V} \sum n(t) \Delta t = \frac{K}{Q_b}$$  \hspace{1cm} (6.6)

where $Q_b$ is the volumetric flow-rate through detector $b$. From the above equation, the area under the elution curve is equal to a constant divided by the volumetric flow-rate of the $b$ liquid stream, $Q_b$. The same equations are also applied for detector $a$. 

144
The area, $h$, is calculated using peak analysis software. The fraction of particles in the $b$ stream, $F_b$, is then calculated:

$$F_b = \frac{Q_b h_b}{Q_{total} h_{total}}$$

(6.7)

There are three possible endpoints for the particles injected into the column, either they will elute in the $a$ stream, elute in the $b$ stream, or be retained on the walls of the channel. It therefore follows:

$$F_a + F_b + F_w = 1$$

(6.8)

where $F_w$ is the particle fraction retained on the accumulation wall.

6.4 Results

The magnetophoretic mobility histograms of the immunomagnetically labeled QSC microbeads are presented in Figure 6.3. From this figure, we see that the magnetophoretic mobility of the QSC microbeads is increasing with increasing ABC. Since magnetophoretic mobility is a function of ABC, and magnetophoretic mobility is also an important factor in determining the ideal total volumetric flow-rates in the QMS separator, we would expect that the magnetic separation efficiency would also be a function of ABC.
Figure 6.3: Histogram of the magnetophoretic mobilities of immunomagnetically labeled QSC microbead populations.
Theoretical plots of the fractional recoveries in streams $F_a$, $F_b$, or retained in the walls of the column, $F_w$ were calculated as a function of total flow-rates using a Maple V (Waterloo Maple, Ontario, Canada) computer program. The program reads in paired mobility-frequency data obtain from CTV, data presented in Figure 6.3, and assigns a starting position and trajectory based on the volumetric flow-rate and mobility of each particle. Based on these trajectories, the final position of each particle was calculated. The theory for this computer code is presented in Williams et al., 1999 and is discussed more thoroughly in Hoyos et al., 2000. Figures 6.4, 6.5, and 6.6 are theoretical plots of the recovered fractions of streams $F_a$, $F_b$, and, $F_w$ versus total volumetric flow-rate for four different QSC microbead populations. The ideal separation rate is determined as the flow condition that gives the greatest recovery in the $b$ outlet stream. The ideal separation flow rate for QSC microbeads with a median ABC of 16,000 was 8 ml per minute, 18 ml per minute for the QSC microbeads with a median ABC of 53,000, and 29 ml per minute for the QSC microbeads with a median ABC of 210,000.

Figure 6.7 is a plot of the theoretical and experimental recoveries in $F_a$ and $F_b$ streams for the QSC microbeads with a median ABC of 18,000. The total volumetric flow rate that gave the maximum recovery in $F_b$ was 9 ml per minute. Figure 6.8 is a plot of the theoretical and experimental recoveries in $F_a$ and $F_b$ streams for the QSC microbeads with a median ABC of 53,000. The total volumetric flow rate that gave the maximum recovery in $F_b$ was 20 ml per minute. Figure 6.9 is a plot of the theoretical and experimental recoveries in $F_a$ and $F_b$ streams for the QSC microbeads with a median ABC of 156,000. The total volumetric flow rate that gave the maximum recovery in $F_b$ was 29 ml per minute.
Figure 6.4: Theoretical recoveries of immunomagnetically labeled QSC microbeads with median ABC of 16,000.

Figure 6.5: Theoretical recoveries of immunomagnetically labeled QSC microbeads with median ABC of 53,000.
Figure 6.6: Theoretical recoveries of immunomagnetically labeled QSC microbeads with median ABC of 210,000.

Figure 6.7: Experimental and theoretical recoveries of immunomagnetically labeled QSC microbeads with median ABC of 18,000.
Figure 6.8: Experimental and theoretical recoveries of immunomagnetically labeled QSC microbeads with median ABC of 53,000.

Figure 6.9: Experimental and theoretical recoveries of immunomagnetically labeled QSC microbeads with median ABC of 156,000.
In comparing experimental and theoretical results from these plots, we see that the theory appears to correctly predict separation flow rates which give maximum recovery in $F_b$ as indicated by the agreement between both theoretical and experimental data, but that the theory underestimated the separation purities for the QSC microbeads with a median ABC of 18,000 (Figure 6.7) and overestimated the separation purities for the QSC microbeads with a median ABC of 53,000 and 156,000 (Figures 6.8 and 6.9).

Figure 6.10 is a linear plot of these separation flow rates that give maximum recovery in $F_b$ versus the ABC of each microbead population. The error bars around the experimental data were estimated based on the approximate range of the nearby data. This plot shows the very close agreement between theoretical and experimental separation flow rates that give maximum recovery in $F_b$ and also shows that optimum separation flow rates are highly dependent upon ABC.

![Figure 6.10: Linear plot of the total volumetric flow rate which gives the maximum recovery in $F_b$ versus ABC.](image-url)
6.5 Discussion

Although the theory accurately predicts the optimal separation flow conditions, it does not accurately predict the particulate recoveries. In both Figures 6.8 and 6.9, the recoveries in outlet stream \( b \) were overestimated and the recoveries in outlet stream \( a \) were underestimated. These comparisons seem to indicate that the flow system has a greater resistance for the magnetized particles to cross than the theory has allowed. This over- and underestimation of the recoveries in outlet streams \( a \) and \( b \) also agrees with other experiments where micron-sized magnetic particles were pulse-injected into the same quadrupole magnetic flow sorter. [Hoyos et al., 2000]

The analysis of the pulse-injection of immunomagnetically labeled QSC microbeads into our quadrupole continuous magnetic cell sorter data has shown that optimal volumetric flow conditions are highly dependent and proportional to the ABC of the microbeads. This information can be extended to cellular systems as well. Cells with higher ABC values are also expected to be labeled with more magnetic material and therefore will require larger flow rates for optimal separation than cells with lower ABC values. With prior knowledge of the ABC of a particular cell sample, the separation conditions can be more finely tuned for each cell separation run. This should enhance separation efficiency by providing information about the sample and alleviate one the problems in cell sample-to-sample variations.
CHAPTER 7

ANALYSIS OF CD34+ ANTIGENS AND OTHER HEMATOPOIETIC ANTIGENS

7.1 Introduction

The CD34 antigen is a heavily glycosylated type I transmembrane protein expressed on early hematopoietic stem and progenitor cells, virtually all small-vessel endothelial cells, and embryonic fibroblasts. (Krause et al., 1996) This antigen is expressed on approximately 1-3% of normal bone marrow cells, 0.01-0.1% of peripheral blood cells, and 0.1-0.4% of cord blood cells. (Stella et al., 1995) The earliest hematopoietic progenitors (CD34+ CD33- HLA-DR-) are small lymphocyte-like cells that lack cytoplasmic granules and prominent nucleoli. This morphology indicates low protein synthesis and low proliferative activity. (Stella et al., 1995)

The CD34+ cells include colony-forming unit macrophages (CFU-M), CFU-granulocytes (CFU-G), CFU-GM, burst-forming units-erythroid (BFU-E) and earlier colony-forming cells CFU-mix and CFU-blast. (Krause et al., 1996) There are two distinct populations of CD34+ bone marrow cells differing in their CD34 surface expression levels, CD34^{bright} and CD34^{dim}. The CD34^{bright} population contains the
majority of the immature hematopoietic progenitor cells and CD34$^{\text{dim}}$ includes more lineage-committed progenitors. CD34$^{\text{bright}}$ cells include unipotent progenitors CFU-GM, BFU-E, CFU-E, CFU-megakaryocyte (CFU-Meg) and early multipotential CFU-GEMM (CFU-mix), CFU-blast, and long-term culture initiating cells (LT-CIC). (Krause et al., 1996) These two distinct populations represent the maturity stage of the hematopoietic cells. Another antigen has been found to correlate with cell maturation independent of cell lineage. This is the CD38 antigen. As a hematopoietic cell matures, its expression of CD34 will decrease, but its expression of CD38 increases. Thus, CD34$^{\text{bright}}$ CD38$^{\text{dim}}$ cells represent the most immature progenitor cells. (Sovalat et al., 1998) This CD34 expression decreases for maturing hematopoietic cell and become undetectable when the hematopoietic cells lose their capacity to form colonies in vitro. For this reason, it has been hypothesized that the CD34 inhibits the development of hematopoiesis. (Krause et al., 1996)

The function of CD34 is still uncertain. It shows no homology to other proteins, but displays weak similarities to cell adhesion molecules. It is thought to play a role in leukocyte adhesion and homing during the inflammatory process and stem/progenitor cell localization/adhesion in the bone marrow and inhibition of hematopoiesis. (Krause et al., 1996) Since the CD34 antigen is a substrate for phosphorylation by protein tyrosine kinase, it may play a role in transmembrane signaling. Although the mechanisms are not yet known, the regulation of the CD34 expression occurs at both the transcriptional and posttranscriptional levels. There has been some early work in whether dysfunctional regulation of CD34 expression plays a role in leukemogenesis. Approximately 40% of
the acute myeloid leukemia cells express CD34. Some have suggested classifying such leukemias as “stem cell leukemias”. (Krause et al., 1996)

Several different anti-CD34 antibodies have been raised against the acute myelogenous leukemia-derived KG1 or KG1a cell lines. The first CD34 antibody, My10 was derived from a hybridoma generated from a mouse immunized with the KG2a myeloid leukemia cell line. (Civin et al., 1987) Other anti-CD34 antibodies include BL3C5, 12.8, 115.2, ICH3, and TUK3. (Andrew et al., 1986 and 1989, Civin et al., 1987, Krause et al., 1996, and Sovolat et al., 1998) The QBEND10 anti-CD34 antibody was raised against human placental endothelial cells. (Krause et al., 1996) An estimate of the number of CD34 molecules per CD34+ bone marrow cell is given as 50,000 My-10 molecules per My-10+ normal human bone marrow cell. This estimate was accomplished by comparing the relative fluorescence of anti-My-10 marrow cells and anti-Leu3a-labeled lymphocytes. This study also found that the My-10 expression is much higher on the surfaces of KG-1a leukemia cells than on normal human marrow cells. (Civin et al., 1987)

All CD34 antibodies specifically recognize the CD34 antigen, but not necessarily the same epitope. (Stella et al., 1995) The CD34 antigen contains a large number of O- and N- glycosylation sites that create different glycosylation dependent epitopes on the antigen. (Sovalat et al., 1998) Monoclonal antibodies recognizing different CD34 epitopes are classified by the sensitivity of the epitope to enzymatic cleavage with neuraminidase from *Vibrio cholerae* and glycoprotease from *Pasteurella haemolytica*. Class I antibodies detect class I epitopes on CD34, which are sensitive to cleavage with neuraminidase and glycoprotease enzymes. Class II epitopes are sensitive to degradation
with glycoprotease but resistant to neuraminidase. Class III epitopes are resistant to the action of both enzymes. (Sovalat et al., 1998, Stella et al., 1995) Since glycoproteases from Pasteurella haemolytica are known to specifically cleave only proteins containing sialylated O-linked glycans, the class III epitopes are thought to be more proximal to the extracellular side of the cell membrane. (Stella et al., 1995)

One study investigated and compared the efficiency of different monoclonal antibodies from the different classes: My10 class I, Qbend10 class II, 8G12 class III, and a mixture of class I and class II monoclonal antibodies called CD34Pool. [8] A lower percentage of CD34+ cells from 13 bone marrow samples was observed with My10 class I (1.24) than with Qbend10 class II (1.56), CD34Pool class I&II (1.84) or 8G12 class III (1.83). Also, the lowest CD34 expression level was observed with My10 (39,000 Ag sites/cell). Slightly higher expressions were observed with Qbend10 and CD34Pool (56,000 and 57,000). The highest expression level was observed with 8G12 (61,000).

The expression of the two different subpopulations of CD34+ cells were found to be approximately 21,000 for the CD34_{dim} and between 63,000 and 84,000 for the CD34_{bright} subpopulation depending on which antibody was used.

My investigations into the expression of CD34 antigens on hematopoietic progenitor cells obtained from apheresis cancer patients were aimed at quantifying the CD34 numbers on these cells and then potentially use this information to look for correlations with either the patient disease, stage of disease, CD34+ cell percentages in blood, G-CSF treatment and/or history of chemotherapy treatment. The accumulated data is reported here, but is not sufficient for any biostatistical significance. Since CD33 and CD38
antigens are also considered markers for the maturity of CD34+ cells, these markers were analyzed along with the CD34 cells in the later studies.

7.2 Materials and Methods

7.2.1 Cell Preparation

A 10 ml sample of apheresis blood product from GM-CSF treated patients was obtained from the Bone Marrow Transplant Lab at the Cleveland Clinic Foundation (Cleveland, OH). The blood cells were washed with 20 ml of wash buffer (Ca- and Mg-free PBS with 0.5 % BSA and 2 mM EDTA, degassed) and counted on a Coulter Counter (Coulter Corporation, Miami, FL). 50 million cells were placed in each tube for immunofluorescent labeling. After labeling with the appropriate reagents, the red blood cells were lysed with 2 ml of Red Blood Cell Lysing Buffer (Sigma Chemical Co., St. Louis, MO) for 10 minutes each.

7.2.2 Quantum Simply Cellular® Microbeads

The Quantum Simply Cellular® (QSC) microbeads used in this study were uniform, 8.0 micron polystyrene microbeads with calibrated numbers of goat anti-mouse antibodies (GAM) bound to their surfaces. Individual sets of these microbeads are coated with four distinct populations of GAM antibodies that bind the Fc region of IgG1, IgG2a, and IgG2b isotypes of mouse monoclonal antibodies and one blank microbead population for controls. The QSC microbeads used in this study were shipped in one vial (lot
#A050699) having uniform antibody binding populations of median ABC of approximately 0, 5,500 18,000, 51,000, and 150,000.

### 7.2.3 Antibody Saturation Studies

Experiments were conducted to insure that the concentration of antibody reagent used in immunolabeling cells and microbeads was saturating all of the available antigen binding sites. To insure this, the cells and microbeads were incubated with different volumes of fluorescent-conjugated antibody following the protocols below.

### 7.2.4 Immunofluorescent Labeling the Microbeads

Approximately 100 µl of the QSC microbead suspension (lot# A-050699) and 100 µl of PBS were placed together in four 15 ml centrifuge tubes each. 80 µl of the primary anti-CD38 PE (lot#14514), 80 µl of anti-33 FITC (lot#15137) and 80 µl of anti-CD34 PerCP (lot#13862) antibody reagent (Becton-Dickinson, San Jose, CA) were added to one tube and to each of the three control tubes for 3-color compensation. These microbeads were incubated with the appropriate antibodies for one hour at room temperature in the dark. These microbeads were then washed twice with 2 ml of PBS, resuspended in 300 µl of PBS and analyzed immediately.
7.2.5 Immunofluorescent Labeling the Cells

The washed cells were placed in 6 tubes of approximately 50 million cells each along with 500 µl of wash buffer. Cells were blocked by adding 50 µl of human IgG blocker (Miltenyi Biotech). 100 µl of anti-CD38 PE (lot#14514), anti-33 FITC (lot#15137) and anti-CD34 PerCP (lot#13862) antibody reagent (Becton-Dickinson, San Jose, CA) was added one tube and to individual control tubes (3 two-color and 1 three-color tube) and allowed to incubate for 30 minutes at 7°C. One tube was left unstained. Cells were then washed with 3-5 ml of PBS, red blood cell lysed, resuspended in 300 µl of wash buffer, and then taken for FCM (FacScan) analysis.

7.2.6 Immunomagnetic Labeling QSC Microbeads with CD34+ MACS Isolation Kit

One ml of these each ABC population of these microbeads (approximately 2 million microbeads) was placed in microcentrifuge tubes. Saturating amounts (250 µl) of primary anti-CD34 hapten (lot#5000228001, Miltenyi Biotech) antibody was added to each tube and incubated for one hour in the dark at room temperature. These microbeads were then washed once with 2 ml PBS and resuspended in 500 µl of PBS. 200 µl of anti-hapten MACS (lot#5991220033) was added and incubated with the microbeads for 30 minutes in the dark at room temperature. The microbeads were then washed twice with 2 ml of PBS each and resuspended in 750 ml PBS for CTV analysis.
7.2.7 Immunomagnetic Labeling QSC Microbeads with CD34FITC and anti-FITC MACS

One ml of these each ABC population of these microbeads (approximately 2 million microbeads) was placed in microcentrifuge tubes. Saturating amounts (300 μl) of primary anti-CD34 hapten (lot#15088, Becton Dickinson) antibody was added to each tube and incubated for one hour in the dark at room temperature. These microbeads were then washed once with 2 ml PBS and resuspended in 500 μl of PBS. 200 μl of anti-FITC MACS (lot#59991018023) was added and incubated with the microbeads for 30 minutes in the dark at room temperature. The microbeads were then washed twice with 2 ml of PBS each and resuspended in 750 ml PBS for CTV analysis.

7.2.8 Cell Separation

Approximately 10 ml of apheresis product from cancer patients treated with GM-CSF was obtained from the Bone Marrow Transplant Laboratory at The Cleveland Clinic Foundation on the same day as the experimentation. These cells were washed in 20 ml of wash buffer (degassed Ca- and Mg-free PBS with 0.5% BSA and 2mM EDTA). The cells were then counted using a Coulter Counter. Of these cells, 150 million were transferred to another tube and wash again with 5-10 ml of wash buffer. This cell sample was suspended in 450 μl of wash buffer and 150 μl of both reagent A1 and A2 from MACS CD34 Isolation Kit (Miltenyi Biotech) and allowed to incubate for 15 minutes at 7 °C and then washed again with 5-10 ml of wash buffer. The cells were then suspended
in 600 µl of reagent B from the MACS CD34 Isolation Kit and incubated for 15 minutes at 7 °C and then washed with 5-10 ml of wash buffer. The final antibody incubation, the cells were suspended in 375 µl of wash buffer and 150 µl of anti-CD34 (HPCA-2) FITC (Becton Dickinson) and incubated for 30 minutes at 7 °C. The cells were then washed twice with 5-10 ml of wash buffer, suspended in 13.5 ml of wash buffer and the total cell concentration was determined using a Coulter Counter. This cell suspension was then immediately processed in our continuous dipole magnetic cell sorter as previously described (Sun et al., 1998). All separation inlet and outlet streams were collected, counted with a Coulter Counter, red blood cell-lysed with 2 ml of Red Blood Cell Lysing Buffer (Sigma Cell Culture) for 10 minutes, and then fixed with 150 µl of wash buffer and 50 µl of 2% para-formaldehyde for next day FACS analysis.

7.3 Results

The immunofluorescently labeled QSC microbeads were analyzed by FCM. A plot of the fluorescence intensities for increasing ABC microbead populations is seen in Figure 7.1. Notice that there are five distinct peaks of fluorescence intensities correlating with the five ABC populations. The median FI values are then plotted against the median ABC of each microbead population in order to obtain a linear relationship between the two values. Before the calibration plot can be obtain, though, we must be certain that we are using the correct amount of antibody reagent to saturation all of the antigen sites on the QSC microbead.
Figure 7.1: Fluorescence intensities of QSC microbeads labeled with anti-CD34 FITC antibody reagent.

Figure 7.2 is a linear plot of the median FI values versus ABC for QSC microbeads labeled with anti-CD33 FITC antibody reagent. The four lines of data represent the four volumes of antibody reagent that was added to the micobeads. Notice that the FI values of each line increase with increasing volumes of antibody reagent, but that the FIs of the 80 μl volume is very close to the 100 μl volume. This was then chosen as the “saturating” volume of CD33 FITC antibody reagent. This experiment was also repeated for CD38PE antibody reagent in Figure 7.3 and CD34 PerCP antibody reagent in Figure 7.4. The results appear very similar for all plots.
Figure 7.2: Median Fluorescence Intensity (FI) versus ABC of QSC microbeads labeled with anti-CD33 FITC antibody reagent.

Figure 7.3: Median Fluorescence Intensity (FI) versus ABC of QSC microbeads labeled with anti-CD38 PE antibody reagent.
Figure 7.4: Median Fluorescence Intensity (FI) versus ABC of QSC microbeads labeled with anti-CD34 PerCP antibody reagent.

The saturation study using all three of the antibody reagents was repeated for the apheresis cell product. These results are seen in Figures 7.5, 7.6, and 7.7 for antibody reagents CD33 FITC, CD38 PE, and CD34 PerCP. Notice that the typical trend is for the FI to increase with increasing volumes of antibody, and that eventually the FI will value plateau. This is the point at which the antibody is saturating the available antigen sites. Further increase in FI after the FI versus volume relationship plateaus is possibly due to non-specific binding of the antibody, and considered undesirable. The median FI in these
plots is taken from the median FI of the positive peak in each plot from FCM. One example of such a plot is seen in Figure 7.8. This plot shows the CD34+ cell population in apheresis blood product. Notice that the majority of the cells do not express the CD34 molecule, but that a small population of cells are more fluorescent, thus express the CD34 antigen. It is the median FI of this positive peak that is of interest.

**Figure 7.5:** Median fluorescence intensities of CD33+ cells versus volume of anti-CD33 FITC antibody reagent.
Figure 7.6: Median fluorescence intensities of CD38+ cells versus volume of anti-CD38 PE antibody reagent.

Figure 7.7: Median fluorescence intensities of CD34+ cells versus volume of anti-CD34 PerCP antibody reagent.
Figure 7.8: Fluorescence intensities of cells labeled with CD34 FITC antibody reagent.

In attempts to quantitate the expression levels of the CD33, CD38, and CD34 antigens, the median FI of each of these cell populations is needed along with calibration plots relating ABC to FI. Using the above mention saturating amounts of each of the antibodies for QSC microbeads, the calibration plots for these three antibodies are obtained. Figures 7.9, 7.10, and 7.11 are the calibration plots of median FI versus ABC for QSC microbeads labeled with saturating amounts of the three anti-CD33 FITC, anti-CD38 PE, and anti-CD34 PerCP antibodies.
Figure 7.9: Median fluorescence intensity versus ABC calibration plot of QSC microbeads labeled with anti-CD33 FITC antibody reagent.

Figure 7.10: Median fluorescence intensity versus ABC calibration plot of QSC microbeads labeled with anti-CD38 PE antibody reagent.
For quantitation of CD33, CD34, and CD38 antigens, the QSC calibration microbeads are run along with FI analysis of the immunofluorescently labeled cell samples. The FI of each color is correlated with its appropriate ABC. Table 7.1 is the accumulated data of these analyses for 13 different patient samples. Only the CD34 antigen was analyzed for the first 6 patients samples, then CD33 and CD38 antigens were also included in the analysis. The purity of CD34+ cells in GM-CSF stimulated patients ranged from 0.2% to 4.5% and the ABC ranged from 46,000 to 101,000 with the average at approximately 80,000. This range in ABC is particularly important in continuous immunomagnetic cell
separation as seen in the work in chapter 5. In addition, the ABC analysis of the CD34+ cell population, it is also interesting to look at the subpopulations of CD34+CD33- and CD34+CD38- cells. The subpopulation of CD34+CD33- cells ranged from 16% to 54% whereas the subpopulation of CD34+CD38- cells was much lower 0.1% to 0.6%.

Combined with the CD34+ antigen analyses, continuous separation experiments were also conducted. The goal of these separation experiments was to optimize the magnetic separation purities and recoveries of the CD4+ cells from apheresis blood product. The data from these separation experiments is listed in Table 7.2. The feed concentration remained relatively constant at approximately 10 million cells per ml, but the feed purity ranged from 0.6% to 3.4%. The two main parameters investigated were the total volumetric flow rate and the transport lamina (or distance of resistance for the magnetized cells to cross into the outer b stream.) The volumetric flow rates tested ranged from 3 to 12 ml per minute and the transport lamina ranged from 206 to 374 μm. The results from these studies are somewhat inconclusive. The purities in b ranged from 3% to 67%, the enrichment rate, a more representative value of the efficiency of the separation, ranged from 5% to 117%, and the total CD34 recovery ranged from 53% to 114%. Obviously, there is a significant amount of error introduced into these combined calculations. An analysis of this error would be important for future work.

Ideally, I would have liked to analyze the ABC of the CD34+ cell populations using immunomagnetics and CTV analysis in addition to immunofluorescence and FCM analysis, but this was more challenging due to the very low purities of the CD34+ cells. I did complete the calibration data using QSC calibration microbeads and two
Table 7.1: List of results from FACS analysis of antigens on hematopoietic cells
<table>
<thead>
<tr>
<th>Date</th>
<th>Q_{\text{total}}</th>
<th>Transport lamina (um)</th>
<th>Feed V ml</th>
<th>Feed C N/ml</th>
<th>Feed Purity %</th>
<th>b1 Purity %</th>
<th>Enrichment %</th>
<th>Cell Recovery %</th>
<th>CD34 Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5/11/99</td>
<td>6</td>
<td>206.4</td>
<td>3.05</td>
<td>2.95E+07</td>
<td>1.89</td>
<td>67.33</td>
<td>35.6</td>
<td>87.5</td>
<td>70.61</td>
</tr>
<tr>
<td>5/11/99</td>
<td>3</td>
<td>206.4</td>
<td>3.25</td>
<td>2.95E+07</td>
<td>1.89</td>
<td>15.41</td>
<td>8.2</td>
<td>86.2</td>
<td>72.23</td>
</tr>
<tr>
<td>5/13/99</td>
<td>5</td>
<td>206.4</td>
<td>2.88</td>
<td>3.95E+07</td>
<td>0.76</td>
<td>11.96</td>
<td>15.7</td>
<td>98.2</td>
<td>54.22</td>
</tr>
<tr>
<td>5/13/99</td>
<td>8.5</td>
<td>206.4</td>
<td>3.03</td>
<td>3.95E+07</td>
<td>0.76</td>
<td>5.28</td>
<td>6.9</td>
<td>82.2</td>
<td>53.92</td>
</tr>
<tr>
<td>5/25/99</td>
<td>3</td>
<td>206.4</td>
<td>3.11</td>
<td>2.72E+07</td>
<td>1.06</td>
<td>33.69</td>
<td>31.8</td>
<td>68.2</td>
<td>78.55</td>
</tr>
<tr>
<td>5/25/99</td>
<td>6</td>
<td>206.4</td>
<td>3.17</td>
<td>2.72E+07</td>
<td>1.06</td>
<td>34.18</td>
<td>32.2</td>
<td>69.4</td>
<td>74.2</td>
</tr>
<tr>
<td>5/25/99</td>
<td>12</td>
<td>206.4</td>
<td>2.18</td>
<td>2.72E+07</td>
<td>1.06</td>
<td>33.13</td>
<td>31.3</td>
<td>50.4</td>
<td>64.01</td>
</tr>
<tr>
<td>5/27/99</td>
<td>5</td>
<td>293.6</td>
<td>5.36</td>
<td>1.18E+07</td>
<td>0.63</td>
<td>74.26</td>
<td>117.9</td>
<td>103.6</td>
<td>92.58</td>
</tr>
<tr>
<td>6/8/99</td>
<td>5</td>
<td>293.6</td>
<td>4.79</td>
<td>1.65E+07</td>
<td>0.64</td>
<td>3.42</td>
<td>5.3</td>
<td>112.4</td>
<td>114.73</td>
</tr>
<tr>
<td>6/29/99</td>
<td>7</td>
<td>293.6</td>
<td>11.2</td>
<td>1.33E+07</td>
<td>0.88</td>
<td>9.32</td>
<td>10.6</td>
<td>86.5</td>
<td>72.56</td>
</tr>
<tr>
<td>7/7/99</td>
<td>5</td>
<td>374.9</td>
<td>9.8</td>
<td>1.15E+07</td>
<td>0.74</td>
<td>31.4</td>
<td>42.4</td>
<td>89.9</td>
<td>78.29</td>
</tr>
<tr>
<td>7/27/99</td>
<td>7</td>
<td>293.6</td>
<td>9.7</td>
<td>1.25E+07</td>
<td>0.31</td>
<td>33.01</td>
<td>106.5</td>
<td>107.2</td>
<td>129.61</td>
</tr>
<tr>
<td>8/24/99</td>
<td>6</td>
<td>293.6</td>
<td>7.6</td>
<td>1.27E+07</td>
<td>3.4</td>
<td>35.01</td>
<td>10.3</td>
<td>148.4</td>
<td>87.29</td>
</tr>
<tr>
<td>8/31/99</td>
<td>6</td>
<td>334.9</td>
<td>9.15</td>
<td>9.24E+06</td>
<td>0.63</td>
<td>21.16</td>
<td>33.6</td>
<td>101.7</td>
<td>105.04</td>
</tr>
</tbody>
</table>

**Table 7.2:** Table listing the separation results of apheresis product immunomagnetically labeled with antibody reagent in MACS CD34 Isolation Kit.

Immunomagnetic antibody labeling schemes: anti-CD34 (HPCA-2) FITC primary antibody with anti-FITC MACS secondary antibody and antibody reagents A2 and B from MACS CD34+ Isolation Kit. Figure 7.12 is a plot of the mean magnetophoretic mobilities of each of these labeled microbead populations versus their corresponding ABC values. Most notable is that the anti-CD34 (HPCA-2) FITC primary antibody with anti-FITC MACS secondary antibody labeling scheme reported much higher magnetophoretic mobilities compared with the antibody reagents from the MACS CD34+ Isolation Kit. This is most probably due to the higher level of secondary antibody amplification ($\psi$) of the anti-CD34 (HPCA-2) FITC primary antibody with anti-FITC MACS secondary antibody labeling scheme (discussed thoroughly in chapter 4).
**Figure 7.12:** Magnetophoretic mobilities versus ABCs for QSC calibration microbeads immunomagnetically labeled with anti-CD34 FITC and anti-FITC MACS reagents and the antibodies from MACS CD34+ Isolation Kit.
7.4 Discussion

The main goals of this analysis of the CD34 antigen expression levels were to quantitate the CD34 antigen expression levels using both FCM and CTV methods of analysis and to use this information of investigate possible correlations of the CD34 antigen expression with patient histories and/or diagnosis. Since this type of study requires a very large sample population, the data reported in Table 7.1 is not yet sufficient to complete this long-term investigation. I hope that this data may be used for its intended purpose at some later date. This analysis did show that the approximate ABC for the CD34 antigen expression was around 80,000, but can range from 46,000 to 101,000.

Additional analysis of the subpopulations of the most immature CD34+ cells, i.e. CD34+ CD33- and CD34+CD38- cells, reported very low levels of the most immature CD34+CD38- cells and higher numbers of the moderately immature CD34+ CD33- hematopoietic progenitor cells. Some preliminary data obtained by Diane Leigh (not shown) using human umbilical cord blood rather than apheresis product from GM-CSF stimulated patients showed slightly higher numbers of the most immature CD34+CD38- cells concluding that human umbilical cord blood may be a better source for true “stem cells” than cells from apheresis product of GM-CSF stimulated patients.
CHAPTER 8

CONCLUSIONS AND FUTURE WORK

8.1 Conclusions

The ability to isolate pure cell populations from a heterogeneous population of cells is important in the diagnosis and treatment of disease as well as having applications in tissue engineering. Currently, state-of-the-art non-batch, continuous immunomagnetic cell sorting devices are under development in the laboratories of Jeff Chalmers, The Ohio State University, and Maciej Zborowski, The Cleveland Clinic Foundation. These devices have distinct advantages over current separation technology in their capacity for large-scale isolation of cell populations. Immunomagnetic separation processes commonly employ paramagnetic nanoparticles conjugated to antibodies directed against specific cellular molecules. Using these paramagnetic nanoparticles, the volume of paramagnetic reagent bound to a cell will dictate that cell’s magnetophoretic mobility when it is placed in an external magnetic energy gradient.

Since knowledge of the degree of immunomagnetic labeling is a central consideration in the design and operation of continuous immunomagnetic cell separation, an understanding of the antibody binding mechanisms, used to bind paramagnetic material to the cell, is critically important. One of the goals of the work in this thesis has focused
on quantitation of the antibody binding mechanisms involved in immunomagnetically labeling cells. Through this work, I have made significant advancements in our understanding of immunomagnetic antibody binding and have developed and published mathematical models describing the fundamental parameters controlling immunomagnetic antibody binding. One of the parameters governing the degree of immunomagnetic labeling and thus, the magnetophoretic mobility of an immunomagnetically labeled cell is called the “antibody binding capacity” or ABC. ABC, related to the number of target cellular antigen molecules, provides quantitative information about the number of primary antibodies binding to the surface molecules on individual cells. I have shown that magnetophoretic mobility is directly proportional to ABC for ABC values less than 30,000 to 50,000. I then used this correlation to quantitate the ABC of a genetically engineered fibrosarcoma model cell line and CD34+ cells from normal human bone marrow, cord blood, and mobilized peripheral blood from patients undergoing autologous stem cell transplantation. I calculated a mean ABC value of 27,000 using CTV analysis and a mean ABC of 33,000 using FCM analysis for the CD2 expression on transformed fibrosarcoma cells. I calculated a range of median ABC values for the CD34 expression on hematopoietic progenitor cells isolated from GM-CSF stimulated patients and found that these median ABC values can range from 46,000 to 100,000, but usually lie at approximately 80,000.

The amount of paramagnetic material bound to a cell is not only a function of the cell’s ABC of the primary antibody, but is also related to the number of secondary antibodies conjugated with paramagnetic material, which bind to epitopes on the primary antibody. In the case where multiple epitopes exist on the primary antibody, several
secondary antibodies may bind per primary antibody. This amplifies the total amount of paramagnetic material bound to the cell and thus would also amplify the magnetophoretic mobility of the target cells leading to a more pure cell separation. By careful manipulation of the experimental conditions, I was able to quantitate the antibody binding amplification of the secondary antibody binding to multiple sites on the primary antibody for two different secondary antibodies specific for two different epitopes on the same primary antibody. Through this work, I have shown that it is possible to obtain amplification in the number of magnetic particles bound to a cell when using an indirect antibody-labeling scheme where the secondary antibody binds to multiple epitopes on the primary antibody. I have developed this original experimental methodology and calculated that approximately 3.4 secondary anti-FITC MACS antibodies bind to each anti-CD4 FITC primary antibody and that approximately 1 secondary anti-mouse MACS antibody binds to each anti-CD4 FITC primary antibody.

In continuous immunomagnetic cell separation, normal patient-to-patient variation of the ABC of a specific subset of cells is a complicating factor in continuous magnetic cell separation. The next goal of my work was to establish the relationship between ABC and magnetic separation efficiency. This relationship was measured by injecting a suspension of immunomagnetically-labeled Quantum Simply Cellular (QSC) calibration microbeads into fluid flowing through a quadrupole magnetic sorter and evaluating the elution profiles of the outlet streams using UV detectors for several separation flow rate conditions. The flow rate producing the best separation was shown to be a function of the ABC, and thus a function of the magnetophoretic mobilities of that microbead population. These elution studies have verified the importance of ABC as it relates to
continuous magnetic separation efficiency and has established a technique for
determining appropriate flow rates for separation of a heterogeneous cell population of
known ABC.

To summarize my contributions to the field of continuous immunomagnetic
separation, I have developed the mathematical theory of the antibody binding used to
impart the paramagnetic material to a target cell, shown experimentally that the ABC of a
cell population is directly proportional to the magnetophoretic mobility of that cell
population, developed methodology for quantitating the ABC and antibody binding
amplification due to secondary antibody binding to multiple sites on the primary antibody
and quantitated the antibody binding amplification of two different secondary antibodies
specific for two different epitopes on the primary antibody. I have also developed
methodology for calculating the ABC of a cell population using immunomagnetics and
CTV technology and shown how this information may be used to set critically important
separation flow rates on our continuous magnetic separation devices.

8.2 Future Work

When antibodies bind to binding sites, these binding sites can be either specific, \( n_s \),
exhibiting a higher binding affinity, or nonspecific, \( n_{ns} \), which typically exhibit a
comparably lower binding affinities. These nonspecific antibody bindings are considered
undesirable binding interactions and typically steps are taken to minimized these as much
as possible. These steps include using a blocking reagent which blocks potential
nonspecific binding sites before the incorporation of the specific binding antibody. It
may be possible to quantitate the number of nonspecific binding sites on a cell. One suggestion is to compare magnetophoretic mobilities for unlabeled and secondary only labeled cells. Any increase in mobility for the cell population labeled with the secondary only antibody would be due to the nonspecific binding. Since magnetophoretic mobility can also be correlated with the ABC of a cell population, the nonspecific binding can be reported in terms of nonspecific ABC. Remembering that:

\[
ABC = (n_s + n_{as})_i \theta_i \lambda_i
\]  

(8.1)

It is also interesting that these antibodies can exhibit different binding affinities depending on the antibody specificity, epitope geometry and amino acid sequence, and the number of epitopes on the specific surface molecule. I have already mentioned that specific binding sites have larger binding affinities than non-specific binding sites. In addition, antibodies can exhibit different binding affinities depending on the number of epitopes on a surface molecule and the binding valence of the antibody binding interaction. These parameters, binding affinity and binding valence, are also important in the overall antibody binding efficiency. In order to completely characterize magnetophoretic mobility, all of these binding interactions need be understood and quantitated.

The last major project that would be beneficial to magnetophoretic mobility characterization and magnetic cell separation optimization would be to design an ideal magnetic particle. In my opinion, this magnetic particle would have the follow attributes: it would composed of a paramagnetic material rather than a ferromagnetic material, this
paramagnetic material would have a high magnetic susceptibility, it would be small in size, approximately 10 to 50 nm in diameter, and the materials in the magnetic particle would be nontoxic such that these could be injected into humans without adverse effects.

The magnetic material in the magnetic particle should be paramagnetic rather than ferromagnetic because the magnetic susceptibility of paramagnetic materials are independent of magnetic field strength, whereas the magnetic susceptibility of ferromagnetic materials are dependent on magnetic field strength, which makes these materials magnetically inducible to some degree and therefore much more difficult to accurately characterize.

Due to steric hindrance issues with larger magnetic particles, it is desirable to have the smallest sized magnetic particles that are realistically possible. This allows the greatest amount of control over magnetic particle binding and magnetophoretic mobility without steric hindrance limitations. Since, in general, we want the immunomagnetically labeled cells to have relatively high magnetophoretic mobilities, in order to obtain an efficient separation, and since we also want very small magnetic particles, we therefore need the magnetic material in these particles to have a relatively large magnetic susceptibility.

Finally, it would be advantageous for these magnetic particles to be nontoxic such that they could be injected into humans with little concern. The overall goal of magnetic cell separation is to isolate cell populations for either analysis for treatment. Researchers isolating cells for analysis would not be as concerned with the toxicity of the magnetic particles, but it is important that these magnetic particles are designed such that they do not limit the end applications of the cell separation technology.
LIST OF REFERENCES


APPENDIX A

PROTOCOLS FOR CELL CULTURE

A.1 Reagents for Fibrosarcoma Growing and Labeling

1. Wash buffer: PBS (Phosphate buffer solution) w/o calcium or magnesium with 1.1% BSA (Note: BSA was shown to have paramagnetic material, thus was left out of CTV experiments.)
2. Re-suspension medium: 10mL FCS (Fetal Calf Serum) and 40 mL PBS
3. Anti-CD2-FITC mouse: Prepare from stock, 1:3 dilution of stock in PBS (20μL of stock per million cells). To a 1.5mL centrifuge tube add 350 μL anti-CD2-FITC and 700 μL wash medium.
4. Anti-FITC-MACS (mouse)beads: Prepare from stock, 1:6.25 dilution of stock MACS beads in PBS (10.67μL stock MACS beads per million cells). To a 1.5mL centrifuge tube add 181μL MACS beads and 952 μL wash medium.
5. Carrier Medium: 4mL of 20mM EDTA solution, fill with PBS to a total volume of 40 mL.
6. 20 mM EDTA solution: Add 3.722g EDTA-dihydrate to 500 mL of PBS.
7. Culture Medium: Dulbecco’s Modified Eagle Medium with 10% Fetal Calf Serum and 1X Penicillin-Streptomycin
8. Phosphate Buffer Saline (PBS): Add the following ingredients to 500 mL double distilled water: 0.1g of potassium chloride, 0.1 g of potassium phosphate monobasic (anhydrous), 0.575 g of sodium phosphate dibasic (anhydrous), and 4 g of sodium chloride. Autoclave on liquid cycle 25 minutes (121°C and 20psig).
9. Freezing Media: 10 mL culture medium and 1 mL sterile DMSO.
A.2 Cell Culture Protocols

A.2.1 Splitting Fibrosarcomas

1. Grow a culture to confluence in a 25 cm$^2$ or 75 cm$^2$ T-flask.
2. Wash twice with 10 mL calcium and magnesium free phosphate buffer solution (PBS).
3. Add ~1 mL Trypsin EDTA.
4. Incubate for 5 minutes @ 37°C.
5. Add 10 mL of culture medium.
6. Wash cells off flask with culture medium.
7. Place cell suspension in 15 mL centrifuge tube.
8. Centrifuge 5 minutes @ 750G, room temperature.
9. Pour off supernatant.
10. Resuspend cell pellet in 10 mL culture medium.
11. Add appropriate fraction to new T-flask. (For a 1:4 split, place 1/4 of cell suspension in each T-flask.)
12. Add 10-15 mL of culture medium to T75 flask (or 5-10 mL to T25 flask).
13. Incubate @ 37°C.
14. Change culture medium when culture medium turns from pink to orange-yellow.
15. Re-split when cells grow to confluence.
A.2.2 Freezing Fibrosarcomas

1. Grow a culture to confluence in several 25 cm$^2$ or 75 cm$^2$ T-flasks.
2. Wash each flask twice with 10 mL calcium and magnesium free phosphate buffer solution (PBS).
3. Add ~1 mL Trypsin EDTA each.
4. Incubate for 5 minutes @ 37°C.
5. Add 10 mL of culture medium each flask.
6. Wash cells off flask with culture medium.
7. Place each cell suspension in a 15 mL centrifuge tube.
8. Centrifuge 5 minutes @ 750G, room temperature.
9. Pour off supernatant.
10. Re-suspend each cell pellet in 1.5 mL freezing media.
11. Add each cell suspension to a 2 mL cryovial.
12. Slowly lower the vials into liquid nitrogen (over 2 hours).
13. Thaw one vial in a few days to check for contamination.

A.2.3 Thawing Fibrosarcomas

1. Remove 1 cryovial from liquid nitrogen.
2. Heat in a 37°C bath until thawed.
3. Place cell suspension in a 15 mL centrifuge tube.
4. Add ~10 mL culture medium.
5. Centrifuge 5 minutes @ 750G, room temperature.
6. Pour off supernatant.
7. Resuspend cells in 10-15 mL culture medium.
8. Add to a T-flask (the same size as was frozen down).
9. Incubate @ 37°C.
A.3 Cell Harvesting and Labeling Procedures for Treated Cells

A.3.1 Harvesting Procedure for IFN-alpha Treated Fibrosarcomas

1. Grow cells to confluence in a 75 cm$^2$ T-flask (Approx. 16 million cells).
2. Split cells from one to two flasks 24 hours prior to IFN treatment.
3. The next day add interferon alpha (IFN-α) to about 12 mL of culture medium to a final concentration of 1500 units/ml. (If the concentration of IFN alpha is 5X10^6 U/ml then you would add 3.6 mL of IFN alpha to 12 mL of media.)
4. Incubate for the desired number of hours @ 37ºC.
5. Remove culture medium.
6. Wash twice with 10 mL of calcium and magnesium free Phosphate Buffer Solution (PBS).
7. Add 10 mL of Versene (PBS + 5mM EDTA) and incubate 5 minutes.
8. Tap on flask to lift cells off surface.
9. Shear cell suspension with a 10 mL pipette.
10. Add suspension to a 15mL centrifuge tube with 3 mL Fetal Calf Serum (FCS).
11. Centrifuge 5 minutes @ 750G, room temperature.
12. Aspirate off supernatant and re-suspend in 10ml culture medium or PBS.
13. Cells are now ready for size and/or count analysis on Coulter Counter or immunomagnetic labeling for CTV analysis.