Investigation of Intrinsic Cell Magnetophoresis for Label-Less Cell Separation and Analysis and the Optimization of the CTV Instrumentation for Such Studies

DISSERTATION

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By

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ABSTRACT

Immunomagnetic cell separation is a rapidly growing technology widely used in the biological research and some clinical applications. It combines simplicity and low cost of separations based on direct application of physical forces to cells with the sensitivity and specificity afforded by use of immunospecific reagents. Current separation strategies and performance highly rely on the availability and choice of the immunomagnetic labels that magnetize cells selected for separation and induce their motion in the magnetic field (magnetophoresis). It is thus of great importance to synthesize and quantitatively characterize immunomagnetic labels for cell separation, which significantly increases cost of magnetic separation and limits its use. An alternative is to develop a label-less magnetic cell separation strategy. Due to low intrinsic magnetic susceptibility of biological material, however, the latter depends on the development of sensitive analytical instruments and methods to characterize the intrinsic cell magnetophoresis and the ability to identify cells with high intrinsic magnetic susceptibility that are significant for diagnostic or therapeutic applications.

In this dissertation, different versions of an experimental instrument, referred to as Cell Tracking Velocimetry, CTV, were developed to measure magnetophoretic mobility (MM) of a micro-sized cell or synthetic particle, either rendered magnetic by tagging or due to their own intrinsic magnetization. MM is a quantitative measure of the magnetic property of a single cell or that of a cell-label complex calculated from the cell motion in
a known magnetic field in a viscous medium. Another analytical apparatus, Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was also employed to measure the intracellular concentration of magnetic metals that contribute to intrinsic cell magnetic susceptibility (iron and manganese). Based on the results of these measurements, candidate “magnetic” cell types were identified and commercial magnetic separators were used for their enrichment from mixed cell samples (red blood cells, RBCs, from hematopoietic cell cultures, apoptotic spermatozoa, and subpopulations of selected cancer cell lines).

The required high accuracy of the CTV instrument was first tested and confirmed by examining the Brownian motion of monodisperse microspheres. The sensitivity of the CTV analysis to different magnetization mechanisms (paramagnetic, diamagnetic and ferromagnetic) on microscale was then investigated. The new and improved, variable-field CTV was shown to be sensitive to the type of the microparticle magnetization and capable of distinguishing between motions of magnetically unsaturated species (paramagnetic or diamagnetic) and the magnetically saturated species (superparamagnetic behavior of ferromagnetic microparticles). Furthermore, with the aid of a fluorescent microscope, the increased functionality of the fluorescent CTV (Fl CTV) was tested and confirmed on cells labeled with fluorescent and magnetic tags (conjugated antibodies). The combination of the regular, visible-light version of CTV with Fl CTV was shown to be able of evaluating the sensitivity and specificity of immunomagnetic labels important for magnetic cell separation.

In the second part of this dissertation, label-less magnetic cell separation was proposed and studied. Cultured RBCs were successfully separated from hematopoietic
stem cell (HSC) bioreactor cultures after deoxygenation by exploiting the paramagnetic nature of deoxygenated hemoglobin. A label-less magnetic selection of nonapoptotic human spermatozoa for in vitro fertilization (IVF) was then investigated and its performance was compared with an established method of immunomagnetic separation of apoptotic cells (expressing phosphatidylin-serine on their surface) labeled with magnetic colloids (annexin V conjugated magnetic nanobeads). The results suggested that apoptotic spermatozoa have a higher intrinsic magnetic susceptibility than nonapoptotic, motile ones. The link to the known elevated intracellular iron and high-spin reactive oxygen species (ROS) in the apoptotic cells has been proposed. Lastly, intrinsic cell magnetophoresis of eight cancer cell lines was examined for potential applications to studies on abnormal intracellular iron metabolism in certain cancers and to circulating tumor cell enrichment and detection. Our results reveal the existence of small subpopulations (1-2%) in K-562 and HeLa cell lines with high intrinsic magnetic susceptibility, further confirmed by their isolation (without labeling) using a commercial magnetic separator. Moreover, the effect of a soluble iron compound addition to cell culture media on the intracellular iron concentration was studied and was found to be different for different cancer cell lines. Such cell metabolism-related soluble iron transfer into intracellular compartment establishes the possibility of an alternative magnetic cell labeling to immunomagnetic labeling. In summary, the result of my studies generated a number of promising and important leads for further investigations of magnetic cell separation in biomedical and biotechnological applications that are now actively pursued in the laboratories of my scientific advisors.
Dedication

This document is dedicated to my family.
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**FIELDS OF STUDY**

Major Field: Chemical and Biomolecular Engineering
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CHAPTER 1

INTRODUCTION

1.1 Classification of materials by magnetic properties

Magnetic susceptibility, $\chi$, is a property of a material describing its response to an applied magnetic field (Cullity 1972). It is equal to the ratio of the magnetization ($M$) within the material to the applied field ($H$), or $\chi = M/H$. The various types of magnetic materials are traditionally classified according to their bulk magnetic susceptibility. Four types are summarized in Table 1.1. Diamagnetic materials are very common, for which magnetic susceptibility, $\chi$, is very small and negative. If a field ($H$) is applied, the electron spins produce a weak magnetization ($M$) that opposes the applied magnetic field. This effect does not saturate and the material has no net magnetic moment when there is no applied field. Water, inert gases, most organic compounds such as cell membrane lipids and nucleic acids, and many metals such as copper and gold are examples of diamagnetic materials.
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<th>Magnetic susceptibility ($\chi$)</th>
<th>Atomic/magnetic behavior</th>
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| Diamagnetism           | Small & negative                | Atoms have no permanent magnetic moment (induced moment only)                           | $\text{H}_2\text{O}: \chi = -9.05 \times 10^{-6}$  
$\text{Au}: \chi = -2.74 \times 10^{-6}$ |
| Paramagnetism          | Small & positive                | Thermally randomized permanent moments are ordered in proportion to the applied field    | $\text{Pt}: \chi = 21.04 \times 10^{-6}$             
$\text{Mn}: \chi = 66.10 \times 10^{-6}$ |
| Ferromagnetism         | Large & positive, complex function of applied field, microstructure dependent.     | Long-distance moment interactions and rise of magnetic domains lead to remnant magnetization (saturation, hysteresis) | $\text{Fe}: \chi = \sim 100,000$                       |
| Superparamagnetism     | Positive                        | Ferro- or ferri-magnetic particles smaller than the magnetic domain (1-10 nm) behave like super-strong paramagnetic molecules | Superparamagnetic iron oxide nanoparticle (SPION)          |

(Adapted from [http://www.aacg.bham.ac.uk/magnetic_materials](http://www.aacg.bham.ac.uk/magnetic_materials))

Table 1.1  Four classes of magnetic materials
A second group of materials for which $\chi$ is small and positive are the paramagnetic materials. The magnetization of a paramagnetic material is weak but is aligned parallel with the direction of the magnetic field. Magnetization is a linear function of the applied field, and varies inversely with temperature, a relationship known as the Curie law (Bozorth 1947). Examples of paramagnetic materials are manganese, platinum and deoxyhemoglobin.

The most important class of magnetic materials is that of the ferromagnetic materials for which the susceptibility is positive, and usually has values $\chi \approx 50$ to 10,000. The important characteristics of a ferromagnetic material are dependence of magnetization on both the applied field and the previous magnetization history (hysteresis), magnetization saturation as the field is increased, and the presence of magnetic domains spontaneously magnetized. Iron and ferrite are examples of ferromagnetic materials.

Superparamagnetism is a magnetic property of nanostructured materials, which consist of individual magnetic domain of elements. As in the case of paramagnetic material, superparamagnetic material as a whole is not magnetized except in an externally applied magnetic field. However, its magnetic susceptibility is much higher than that of paramagnetic material. Ferritin and many commercially available iron oxide nanoparticles belong to this group. They exhibit saturation magnetization at relatively weak fields.
1.2 Magnetophoresis and immunomagnetic cell separation

Magnetophoresis refers to the movement of microparticles relative to the suspending solution under the influence of a non-homogeneous magnetic field. The term was proposed in analogy to electrophoresis (an electric field-induced motion) (Hartig et al. 1992; Leenov 1954; Watarai et al. 2004; Winoto-Morbach et al. 1994). The difference in magnetic susceptibility between different cell subsets suspended in viscous aqueous media, which results in the difference in magnetophoresis, establishes a basis for magnetic field-based cell separation and detection (Zborowski et al. 2002). Typically, difference in magnetic susceptibility is imparted through selective binding of magnetizable micro- or nano-particles. This is because in nature, magnetic properties of cells are dominated by the diamagnetic properties of their major components, such as water, proteins, nucleic acids, carbohydrates, and lipids (Chikov et al. 1991; Torbet 1987). Even with the application of high magnetic field, the magnetic force acting on a cell is very small, and it is practically impossible to induce any perceptible motion. Therefore, most cells must be selectively labeled with a magnetic tag before they can be separated.

The selective binding of magnetic tags to cells is achieved using an antigen-antibody reaction or other suitable receptor-ligand reaction, which gives the immunomagnetic separation technology high accuracy and specificity as well as high purity. Compared to fluorescence-activated cell sorting (FACS), another cell separation method utilizing cell immunological specialty which separates cells based on the fluorescence intensity of cells labeled with antibody fluorochrome conjugates (Herzenberg and Sweet 1976), immunomagnetic cell separation has advantages of lower
capital and operating costs, simple and fast separation procedure, and high throughput. Therefore, it has been employed widely in biological research and clinical application, such as selection of rare cancer cells or hematopoietic stem cells (HSCs) from peripheral blood and bone marrow (Choesmel et al. 2004; Chou et al. 2005; Jing et al. 2007; Zigeuner et al. 2000), and depletion of unwanted T-cells from blood samples for allogeneic HSC transplantations (Gordon et al. 2002; Tong et al. 2007).

However, identifying a linking molecule with high specificity for the desired cell is essential for immunomagnetic separation and detection. Therefore, the specificity of the separation and detection depends strongly on cell immunophenotype and use of antibodies that distinguish between characteristic cell surface antigen markers. The magnetic separation apparatus is relatively inexpensive and separation process is simple and fast. However, the cost of the immunomagnetic reagents quickly adds up in the course of just a few separation runs, and the time required for sample preparation by immunomagnetic labeling limits its wide application.

1.3 Magnetic particles and immunomagnetic cell labeling strategies

Currently, both micro- and nano-sized magnetic particles have been used for immunomagnetic labeling and are commercially available. They are typically composed of a core of single-domain (~10 nm) iron oxide particles (usually magnetite, Fe$_3$O$_4$, or maghemite, γ-Fe$_2$O$_3$) coated with a biocompatible polymer such as dextran. They exhibit superparamagnetic behavior that enables their stability and dispersion after removal of
the magnetic field since they do not retain remnant magnetism (Huber 2005; Safarik and Safarikova 1999).

Micro-sized magnetic particles with diameters typically from 1 to 5 µm such as Dynabead® magnetic particles (available in sizes 1, 2.8, 4.5, and 5.0 µm, Invitrogen, Inc., Carlsbad, CA) have high magnetic moment and thus separate the labeled cells quickly in a relatively weak field gradient. They are preferred for negative cell selection, after which the labeled cells are discarded without the need for bead removal manipulations. Nano-sized particles or colloidal particles are typically 50 - 200 nm in diameter and have a relatively low magnetic moment but fast cell binding reaction kinetics. MACS® MicroBeads (~50 nm, Miltenyi Biotec, Germany), BD IMag™ particles (~100 - 450 nm, BD Biosciences, San Diego, CA), and Easysep® magnetic nanoparticles (~150 nm, StemCell Technologies, Inc., Canada) are examples of nano-sized particles with wide applications.

There are two different strategies for labeling cells with magnetic particles, as shown in Figure 1.1. Direct labeling, or one-step labeling, is fast and simple. In this case, magnetic beads are conjugated with antibodies targeting the specific cell surface antigens. However, indirect labeling, or two-step labeling, is more commonly used. This is because it is more flexible and may be used when the antibody of interest coupled to magnetic beads is unavailable, or when a limited magnetic moment is produced by direct labeling because of dimly expressed surface antigens. In indirect labeling, cells are first labeled either with a primary antibody or with a primary antibody that is for instance biotinylated or fluorochrome-conjugated. In a second step, magnetic particles conjugated with a secondary antibody are employed that recognize the primary antibody or the molecules
on the tail of the primary antibody. During two-step labeling, however, cell loss increases and there is a requirement for more washing steps.

![Diagram of magnetic cell labeling](image)

Figure 1.1 Schematic presentation of direct (A) and indirect (B) magnetic cell labeling

1.4 Magnetophoretic mobility

Magnetophoretic mobility (MM) is a quantity introduced to describe the magnitude of magnetophoresis. When a cell or particle in a viscous medium is placed in a magnetic field gradient, the magnetic force is given by the equation

\[ F_m = \Delta \chi V \nabla \left( \frac{B_0^2}{2\mu_0} \right) \]  

(1.1)
where $\Delta \chi = \chi_c - \chi_f$, and $\chi_c$ and $\chi_f$ are the magnetic susceptibilities of the cell/particle and the suspending medium, $V$ is the volume of the cell/particle, and $B_0$ is the applied magnetic field, $\mu_0$ is the magnetic permeability of a vacuum ($= 4\pi \times 10^{-7}$ T·m/A). If the magnetic force is designed to be perpendicular to gravity force, at steady state, it is just counteracted by the drag force, $F_d$, of the continuous medium so that the cell/particle reaches its terminal velocity in microseconds. In the limit of a creeping flow, applicable to the motion of a cell/particle in a viscous medium, the drag force can be assumed to follow the Stokes formula,

$$F_d = -3\pi \eta D u_m$$

(1.2)

where $\eta$ is the viscosity of the medium, $D$ is the hydrodynamic diameter of the cell/particle, and $u_m$ is the terminal velocity of the cell/particle.

At the terminal velocity, the sum of the forces given by eqns (1.1) and (1.2) is zero and one obtains

$$u_m = \frac{\Delta \chi V}{3\pi \eta D} \nabla \left( \frac{B_0^2}{2\mu_0} \right)$$

(1.3)

The above expression indicates that field-induced velocity is a function of two independent quantities, one being the magnetic energy density gradient $S_m = \nabla (B_0^2/2\mu_0)$, the other being a combination of material properties of the cell/particle and the suspending fluid. This suggests a definition of the magnetophoretic mobility, $m$, in an analogous manner to the well known definition of sedimentation coefficient, $s$ ($u_s = s g = \Delta \rho g / 3\pi \eta D$, where $g$ is the standard gravitational acceleration) or electrophoretic mobility, $\mu$ ($u = \mu E$, with $E$ being the electric field strength) (Markx and Pethig 1995). We therefore have
\[ u_m = mS_m \]  

(1.4)

and

\[ m = \frac{\Delta \chi V}{3\pi \eta D} \]  

(1.5)

In one dimension eqn (1.4) simplifies to

\[ m = \frac{u_m}{S_m}, \text{ where } S_m = \frac{d}{dx} \left( \frac{B^2}{2\mu_0} \right) \]  

(1.6)

For most unlabeled cells, the intrinsic MM, given by eq (1.5), is negligible since \( \Delta \chi \approx 0 \). When a cell is labeled with magnetic colloidal beads through a two-step labeling protocol (Figure 1.1), its MM becomes a function of the primary antibody binding capacity, ABC, the secondary antibody amplification (the number ratio of secondary antibodies to primary antibodies; equal to 1 if only a one-step labeling is involved), \( \psi \), the number of magnetic nanoparticle conjugates per secondary antibody, \( n \), and the material properties of the single magnetic nanoparticle and the suspending medium, \((\chi_{ns} - \chi_f)V_{ns}/3\pi \eta D_t\), where \( \chi_{ns} \) and \( V_{ns} \) are the magnetic susceptibility and volume of the magnetic nanoparticle, \( D_t \) is the hydrodynamic diameter of the cell-label complex (McCloskey et al. 2003a),

\[ m = \frac{ABC \cdot \psi \cdot n \cdot (\chi_{ns} - \chi_f) V_{ns}}{3\pi \eta D_t} \]  

(1.7)

MM has been used as a parameter to characterize and evaluate the degree to which a cell is immunomagnetically labeled. This is determined by the selectivity and specificity of the binding of the labeling reagent to and the distribution of external antigens expressed by the specific cell population (Chosy et al. 2003; McCloskey et al. 2000; McCloskey et al. 2001a). The performance of the magnetic cell sorting was shown to be directly
predictable from the MM distribution (Comella et al. 2001; McCloskey et al. 2003b; Melnik et al. 2001; Williams et al. 1999). Thus, MM analysis contributes to the improvement of magnetic labels, labeling methodology, and cell separation instrument design and operation.

1.5 Cell tracking velocimetry (CTV)

Cell tracking velocimetry (CTV) is an analytical technique developed in our group to experimentally measure the MM of micro-sized cells or particles on a cell-by-cell basis (Chalmers et al. 1999c; Moore et al. 2000; Nakamura et al. 2001). A schematic diagram of this instrument is presented in Figure 1.2. The central part of this apparatus is the permanent magnet assembly, with specially designed pole pieces spaced 2.5 mm apart. This produces a nearly constant magnetic energy density gradient, \( S_m (140 \text{ TA/mm}^2, \text{S.I.}) \) units are used throughout this work), in the area where the measurement is made (a rectangular viewing area of 1.72 mm \( \times \) 1.27 mm, where \( B_0 = 1.260 \pm 0.115 \text{ T}, \text{grad} B_0 = 0.140 \pm 0.012 \text{ T/mm} \)). Hence, the horizontal cell velocity \( u_m \) induced by the magnetic force, perpendicular to the settling velocity \( u_g \) induced by gravitation, remains almost constant in the viewing area. The MM is determined from the observed \( u_m \) via eqn (1.6).

The cell suspension to be analyzed is pumped into a rectangular borosilicate glass channel which is placed in the interpolar gap of the magnet as shown in Figure 1.2. The cell motion analysis is performed in the stationary fluid (no convective flow). The displacements of cells are recorded using an inverted microscope and a high-sensitivity, monochrome CCD camera. The captured images are then digitalized into two dimensional matrixes of particle position using an imaging-grabbling board, and the
position information processed by CTV software (Imagview). This produces an Excel™ file containing cell velocities, MM, and sample statistics. The permanent magnet assembly may be replaced with an electromagnet system which not only allows the magnetic field to be cycled on and off, but also produces variable magnetic field strength for different applications.

Figure 1.2 Diagram of the cell tracking velocimetry system.

1.6 Magnetic cell sorters

A number of commercial and homemade magnetic separation systems have been designed for the different types of magnetic particles (micro- or nano-size) used for
different applications. Currently, the commercially available systems operate in batch-wise mode, which are generally unsuited to scale-up. Among the most used and successful are the MACS® systems from Miltenyi Biotec, the DynaMag Systems from Invitrogen, the BD Imag separation systems from BD Bioscience, and the EasySep Systems from StemCell Technologies. Open gradient magnetic separators (OGMS) without a high-permeability wire or microsphere matrix inside are utilized in the latter three systems, and magnetically labeled cells are simply captured on the tube wall by a magnet, followed by removal of the supernatant containing the non-magnetic cells. This type of separator generally has low magnetic field gradient, and results in slow accumulation rates of magnetic cells on the tube wall, particularly in the case of positive selection of rare cells. The MACS system (Figure 1.3) is a typical example of a high gradient magnetic separator (HGMS). Spherical steel matrix is packed in the MACS columns, within which a high gradient magnetic field is induced by applying an external magnetic field. In principle, unlabeled cells pass through the column. Magnetically labeled cells are captured on the surface of the steel spheres and retained in the column. They are subsequently washed out and collected after removal of the magnetic field. This method is fast and relatively efficient especially for small scale cell separation. However, clogging problems can arise due to the deposition of magnetic cells on the matrix, and this limits its separation capacity.
A continuous, flow-through magnetic cell sorter has been developed in the author’s group, referred to as the Quadrupole Magnetic cell Sorter (QMS). It employs a quadrupole magnetic field and annular channel geometry. The theory of split flow thin (SPLITT) fractionation technology developed by Giddings and coworkers (Giddings 1985; Williams et al. 1999) has been adapted for this system. As depicted in Figure 1.4, its separation element consists of two concentric cylinders (the inner one is solid) surrounded by an in-house designed quadrupole magnet assembly, which creates a magnetic energy density gradient increasing linearly in the radial direction from the center to the tip of the pole pieces, and this is independent of the axial and angular directions. Cell mixtures enter the channel through inlet $Q_a$, and carrier fluid enters through inlet $Q_b$. Magnetic cells (red circles in Figure 1.4B) (either intrinsically magnetic or imparted via magnetic labeling) then move radially outward under the influence of the
magnetic field gradient and pass through the transport lamina, while non-magnetic cells (blue circles in Figure 1.4B) move straight along the flow. Therefore, the magnetic and non-magnetic cells can be collected at different outlets. This continuous separation system is easy to scale up.

Figure 1.4 Schematic diagram of the QMS system (A) and Cross section of the channel in operation (B). Red and blue circles represent magnetic and non-magnetic cells, respectively.

1.7 Intrinsic cell magnetophoresis

As stated earlier, the main limitation of immunomagnetic cell separation is the dependence on immunomagnetic labeling itself which increases the process time, raises
the expense, and may influence the separation outcome. A magnetic field-based cell separation and detection method relying solely on the intrinsic magnetic susceptibility of cells would be very attractive. It would require the difference in magnetophoresis between different cell subsets to be sufficiently large under an applied magnetic field gradient. This is difficult to achieve due to the fact that the diamagnetic susceptibility of water and other cellular components is very low as compared to susceptibility of paramagnetic and especially ferromagnetic materials. However, there are a few notable exceptions. Some cells such as magnetotactic bacteria and erythrocytes are rich in magnetic metals.

### 1.7.1 Magnetic prokaryotic cells

Magnetotactic bacteria (MTB) first discovered by R. P Blakemore (Blakemore 1975) in 1975, are capable of synthesizing nano-sized magnetite ($\text{Fe}_3\text{O}_4$) or greigite ($\text{Fe}_3\text{S}_4$) crystals in cell organelles called “magnetosomes” (Bazylinski et al. 1994). They may be removed from wastewater by harnessing their magnetic properties in a orientation magnetic separation process (Bahaj et al. 1998; Bahaj et al. 2002).

Melnik et al. (Melnik et al. 2007) observed that the spores of at least three strains of *Bacillus: Bacillus atrophaeus* (formally *Bacillus globigii*), *Bacillus thuringiensis*, and *Bacillus cereus* exhibited significant intrinsic magnetic susceptibility. All three strains when sporulated demonstrated significant MM using the CTV system. Energy dispersive spectroscopy confirmed that this magnetic susceptibility is the result of the presence of manganese (Mn). A magnetic deposition system was applied to separate the spores exploiting their intrinsic magnetic susceptibility.
1.7.2 Red blood cells

Since the pioneering work by Pauling and Coryell (Pauling and Coryell 1936a; Pauling and Coryell 1936b) on the magnetic susceptibility of red blood cells (RBCs), many investigations have reported the magnetic properties of RBCs incorporating hemoglobin (Hb) in different iron oxidation states (Coryell et al. 1937; Savicki et al. 1984; Zborowski et al. 2003). Hemoglobin, an iron-binding protein, occupies one-third of the RBC volume. It has no unpaired electrons and thus becomes diamagnetic when oxygen binding to the iron atoms of its four heme groups, as covalent bonds formed between the iron atom and the four nitrogens in each heme group, oxygen, and the globin chain. In contrast, deoxygenated hemoglobin (deoxy Hb) and methemoglobin (metHb) in which the iron atom is ionically bound are paramagnetic because of the existence of four and five unpaired electrons, respectively, in each heme group. Therefore, oxygenated RBC (oxy RBC) is more diamagnetic than common aqueous media and deoxygenated RBC (deoxy RBC) and metHb containing RBC are less diamagnetic than the aqueous media. The separation of RBCs directly from white blood cells (WBCs, diamagnetic) in whole blood has been attempted using an HGMS system by either deoxygenation or methemoglobinization treatment (Melville 1975; Owen 1978; Takayasu et al. 2000). Magnetophoretic microsystems were also designed to isolate deoxy RBCs and WBCs based on their native magnetic properties, where a much higher magnetic force compared to the conventional macro scale separators was generated on cells (Furlani 2007; Han and Frazier 2004; Han and Frazier 2006).

In addition, the presence of the paramagnetic hemozoin in malaria infected erythrocytes has been identified by many studies (Bohle et al. 1998; Moore et al. 2006).
As early as 1946, Heidelberger et al. (Heidelberger et al. 1946) attempted to enrich the malaria infected erythrocytes by applying the paramagnetic properties of hemozoin. Recently, a novel method, termed magnetic deposition microscopy (MDM) (Zimmerman et al. 2006) was developed to isolate infected RBCs from a cell suspension and accumulate them on transparent polymer slides suitable for light microscopy. It yielded higher sensitivity than conventional thick blood smears and approaches the sensitivity of the reverse transcriptase polymerase chain reaction (RT-PCR) in detecting the malaria gametocytes in the blood of infected individuals (Karl et al. 2008).

There may be other undiscovered cells having a paramagnetic property caused by sufficient intracellular magnetic metal elements or other molecules. Cancer cells are of particular interest because of potential diagnostic applications. In fact, a number of published reports suggest that iron overload could lead to neoplastic transformation (De Freitas and Meneghini 2001; Huang 2003; Papanikolaou and Pantopoulos 2005).

1.8 Possibility of significant intrinsic magnetic susceptibility in cancer cells

1.8.1 Iron metabolism in cancer

Iron is an indispensable requirement for the activity of many vital biochemical processes, such as oxygen transport, electron transfer, and DNA synthesis (Aisen et al. 2001). Most iron in mammalian cells is bound in the heme group of hemoproteins such as hemoglobin and cytochrome, or a constituent of proteins containing iron-sulfur clusters (Beinert et al. 1997). Transferrin is an important Fe-binding protein in mammalian cells; it binds two iron atoms with high affinity. Extracellular irons are transported by and
released from transferrin intracellularly, mainly through transferrin receptor-mediated endocytosis (Klausner et al. 1983). Excess irons are normally stored in the intracellular Fe storage protein, ferritin. One molecule of ferritin can accommodate up to 4,500 atoms of iron in its internal cavity (Harrison and Arosio 1996).

A growing body of evidence indicates that iron overload is clearly associated with a high risk for carcinogenesis. In 1959, Richmond (Richmond 1959) first reported the iron-induced malignant tumors resulting from repeated intramuscular injection of iron dextran complex in rats. Excessive accumulation of “free” iron in hepatocytes may lead to the development of hepatocellular carcinoma (Bonkovsky 1991; Kew 2008; Nahon et al. 2010). Epidemiological, animal, and cell culture studies were summarized by Huang (Huang 2003) to illustrate the carcinogenic role of iron in the development of a range of cancers including colorectal, liver, kidney, lung and stomach cancers.

The mechanisms for iron-induced neoplastic transformation are poorly characterized. However, it is clear that iron overload disrupts the redox balance of the cell and generates reactive oxygen species (ROS) through Fenton and Haber-Weiss reactions (Fenton 1894; Halliwell and Gutteridge 1992), which then facilitates mutagenesis and modulates signaling networks related to malignant transformation (Benhar et al. 2002; De Freitas and Meneghini 2001; Papanikolaou and Pantopoulos 2005; Waris and Ahsan 2006). ROS are also paramagnetic molecules containing one or more electrons with unpaired spin.

In addition, an elevated level of serum and leukocyte ferritin was reported in patients with leukemia. It was suggested that high serum ferritin concentrations were related to an increased ferritin synthesis and concentration in the circulating leukocytes
(Worwood et al. 1974). Increased serum and/or lymphocyte ferritin synthesis and release was found in patients with Hodgkin’s disease (Sarcione et al. 1977), breast cancer (Moroz and Kupfer 1981) and hepatocellular carcinoma (Melia et al. 1983). Ferritin has been proposed as a clinical marker for staging and predicting survival for renal cell carcinoma, particularly in the case of recurrence after surgical therapy (Miyata et al. 2001).

On the other hand, intracellular iron plays an important role in the proliferation of cancer cells, particularly in DNA synthesis and cell cycle progression. Cancer cells are more likely to express a large number of transferrin receptors 1 (TfR1 or CD71), resulting in higher cellular iron uptake and greater proliferation rate than normal cells (Larrick and Cresswell 1979; Weinberg 1992). It was also found that the c-Myc oncogenic transcription factor stimulates proliferation and growth of cancer cells by activating TfR1 and other molecules involved in iron metabolism (O'Donnell et al. 2006).

*In vitro* studies have also shown that human cancer cells produced larger amounts of ROS than non-transformed cells, which kept cancer cells in persistent oxidative stress (Brown and Bicknell 2001; Okamoto et al. 1996; Szatrowski and Nathan 1991; Toyokuni et al. 1995). Under stress conditions, more “free” iron tends to be released from iron-containing molecules.

### 1.8.2 Manganese and cancer

Besides iron, manganese is another paramagnetic essential metal present in cells, but at much lower concentration than iron. Manganese functions as a constituent of metalloenzymes and as an enzyme activator in animal tissues and cells (Keen et al. 2000).
Manganese-superoxide dismutase (MnSOD), one of important manganese-containing enzymes, is one of the active oxygen scavengers in the mitochondria and is necessary to protect oxygen-utilizing cells from the toxicity of the produced ROS. Increased MnSOD expression has been observed in the experimental ovarian cancer animal model (Hu et al. 2005; Nakata et al. 1992) as well as primary ovarian cancer tissues from patients who respond to intrinsic ROS stress (Hileman et al. 2004). A number of other malignant tumors were found to express higher levels of MnSOD than their non-malignant progenitor cells, such as gastrointestinal malignancies, central nervous system tumors, leukaemia, and melanomas (Kinnula and Crapo 2004).

Overall, the elevated intracellular iron, ferritin, manganese, and ROS may confer significant native paramagnetic susceptibility of some cancer cells relative to the suspending media.

1.9 Possibility of significant intrinsic magnetic susceptibility in apoptotic spermatozoa

Besides causing malignant transformation, excessive free iron and ROS may induce cell apoptosis. Human spermatozoa are especially sensitive to ROS-induced damage due to the high content of polyunsaturated fatty acids in the sperm plasma membrane that readily undergo lipid peroxidation, and the low concentration of scavenging enzymes within the cytoplasm (Jones et al. 1979; Sharma and Agarwal 1996). As a consequence of exposure to oxidative stress and ROS induced apoptosis, sperm membrane is impaired, motility is decreased, and DNA is damaged (Agarwal et al. 2008b; Ollero et al. 2001). It
has been identified as a major factor in male infertility (Agarwal et al. 2003; Sikka 2001). Additionally, the content of manganese in the form of MnSOD, an important antioxidant as discussed earlier, may also increase as a result of oxidative stress. Therefore, the link between sperm cell apoptosis and the presence of ROS and the role of iron and manganese in ROS generation gives rise to an interesting hypothesis that the apoptotic spermatozoa may possess a higher magnetic susceptibility as compared to healthy, motile cells.

1.10 Quantification of intracellular metal elements

The intrinsic magnetic susceptibility of a cell is partially due to intracellular magnetic metal elements. But due to the low level of most metals in cells (fg/cell level) and limited cell numbers in a typical biological sample, a highly sensitive and reliable analytical technique is essential for cellular metal element quantification. Currently, atomic spectrometric techniques are widely used for analysis of trace elements including atomic absorption spectrometry (AAS), inductively coupled plasma-atomic emission spectrometry (ICP-AES) and inductively coupled plasma-mass spectrometry (ICP-MS) (Beauchemin 2006; Bings et al. 2008). Atomic Absorption (AA) occurs when a ground state atom absorbs energy in the form of light of a specific wavelength and is elevated to an excited state. Each metal has a characteristic wavelength that will be absorbed. There is a linear relationship between the energy absorbance and the concentration of the element. By using this relationship in known standards and measuring the amount of light absorbed, the concentration of the same element in an unknown sample can be determined (Haswell 1991). ICP-AES (Montaser and Golightly 1987) is the measurement
of the light emitted by the elements in a sample introduced into an ICP source. Elemental concentrations in the unknown sample are then determined by comparing the measured emission intensities to the intensities of standards of known concentration. ICP-MS (Huang et al. 2006; Woeste 2004) employs ICP as the ionization source to generate singly charged ions from the elemental species within a sample which are directed into a mass spectrometer where they are separated according to their mass-to-charge ratio. Ions of the selected mass-to-charge ratio are then directed to a detector that determines the number of ions present (Figure 1.5). Detection limit ranges for the major atomic spectroscopy techniques are shown in Figure 1.6 (Zhang 2007). ICP-MS is the most sensitive and is capable of detecting most elements at the sub part per billion (ppb) to sub part per trillion (ppt) levels.

Figure 1.5  Schematic of ICP-MS (Shcherbyna et al. 2006)
1.11 Sperm selection for ART

Fifteen percent of all couples in the US are infertile and the male factor is responsible for 25% of these cases (Sharlip et al. 2002). Assisted reproductive techniques (ART) have become the treatment of choice in many cases of infertility. The human ejaculate is comprised of a mixture of seminal plasma, mature and immature spermatozoa, non-reproductive cells (e.g. leukocyte), and debris. The selection of sufficient of the most fertilizable sperms from the surrounding milieu is one of the pre-requisites for achieving successful in vitro fertilization (IVF). Current routine sperm preparation techniques such as swim-up and density gradient centrifugation separate spermatozoa based on their motility or density (Henkel and Schill 2003). The selected sperm quality is evaluated by conventional semen analysis using a light microscope to determine sperm concentration,
motility, and morphology (WHO 1999). Nevertheless, molecular events such as sperm apoptosis are overlooked in the course of routine ART. It has been found that the inclusion of spermatozoa displaying features of apoptosis despite normal appearance during IVF may be partially responsible for the current unsatisfactory ART success rate (Henkel et al. 2004; Seli et al. 2004; Sunderam et al. 2009). Apoptosis is a mode of programmed cell death based on a genetic mechanism, which induces a series of cellular, morphological, and biochemical alterations and then leads the cell to suicide (Vaux and Korsmeyer 1999). Hence, there is the need to develop new protocols for non-apoptotic, motile sperm selection based on the apoptosis marker or property. One of the early apoptotic features in human spermatozoa is the externalization of phosphatidylserine (PS) from its normal location in the inner leaflet of the sperm plasma membrane to the outer surface (Oosterhuis et al. 2000; Vermes et al. 1995). Annexin V has a high affinity to PS (Van Heerde et al. 1995). By conjugating magnetic colloids to annexin V it is possible to use the affinity between annexin V and PS to impart significant magnetic susceptibility to apoptotic spermatozoa. A new separation procedure was reported to yield higher quality of spermatozoa combining the density gradient centrifugation with a MACS separation system, in which apoptotic spermatozoa labeled with annexin V-conjugated microbeads were eliminated (Said et al. 2008; Said et al. 2005). Alternatively, it is possible to deplete apoptotic spermatozoa magnetically, based on their potentially high intrinsic magnetic property.
1.12 Circulating cancer cell enrichment and detection

It is generally accepted that most cancer-related mortalities result from metastatic disease (Leaf 2004). Viable tumor-derived circulating cancer cells (CTCs) have been identified in peripheral blood from cancer patients and are probably the origin of intractable metastatic disease (Cristofanilli 2006; Gupta and Massague 2006; Steeg 2006). These tumor cells leave the primary tumor site, invade the circulatory system, and then migrate to a new site where they form a metastatic tumor (Pantel et al. 1999). CTCs have become an emerging “biomarker” for detecting early-stage cancer metastasis, and predicting patient prognosis, as well as monitoring disease progress and therapeutic outcomes (Mocellin et al. 2006; Mostert et al. 2009; Paterlini-Brechot and Benali 2007). However, detection of CTCs is very difficult due to the extremely low frequency in peripheral blood (as low as one CTC in $10^6 - 10^7$ leukocytes). One or more pre-enrichment steps are normally needed to make the final detection (e.g. ImmunoCytoChemical Staining, ICCS, and Reverse Transcriptase Polymerase Chain Reaction, RT-PCR) practical.

Many different types of enrichment methods in macro or micro scales have been developed employing the differences between CTCs and nucleated blood cells in size, density, surface markers, and so on. The technology of isolation by size of epithelial tumor cells (ISET, Metagenex), allows the collection of CTCs by filtration, as CTCs are larger than blood leukocytes (Vona et al. 2000). However, experience with this method remains limited. Density gradient separation of mononucleated cells (including CTCs) from blood has been obtained using Ficoll (Amersham, Upsala, Sweden), Lymphoprep
(Nycomed, Oslo, Norway) or other similar density gradient liquids. This technique is often combined with magnetic cell separation. Magnetic cell separation is the most widely practiced approach, involving positively targeting CTCs or negatively depleting CD45-expressing peripheral blood leukocytes with magnetic beads. Since specific antigens characterizing CTCs are not known and most CTCs develop from epithelial cells, immunomagnetic antibodies specific to epithelial cell markers such as EpCAM (epithelial cell adhesion molecule) and CK (cytokeratins) are being used to identify CTCs. CellSearch CTC test (Veridex, Johnson & Johnson Company, http://veridex.com/CellSearch/CellSearchHCP.aspx) is the only FDA-approved assay in which CTCs are enriched from whole blood by adding ferrofluids coated with antibodies to EpCAM. However, some CTCs are not of epithelial origin, such as melanoma CTCs (Mocellin et al. 2006), and epithelial cell markers may also be expressed in non-tumorous cells. In contrast, a purely negative enrichment method has been reported in which only normal blood cells were targeted by immunomagnetic reagents and depleted, thereby allowing rare, non-hematopoietic cells to be significantly enriched (Yang et al. 2009). But in either positive or negative magnetic selection, specific immunomagnetic reagents are required.

1.13 Dissertation organization

This dissertation comprises two major parts. The first part of the study was to further develop and perfect CTV instrumentation and protocols. The second part concerned the investigation of intrinsic magnetophoresis of cells and label-less magnetic cell separation.
The outline of Chapter 2 through Chapter 8 is as follows.

Chapter 2 describes the evaluation of the measurement error of the CTV system. Brownian motion of monodisperse standard microspheres was used as an experimental model. The expected and observed variances of cell mobility due to Brownian motion were determined and compared.

Chapter 3 describes for the first time the functional dependence of MM on the applied magnetic field for different types of magnetic microparticles. The MM of linearly polarizable (paramagnetic and diamagnetic) particles and magnetically saturated (superparamagnetic) particles in the variable magnetic field were determined by experimental analysis using variable-field CTV and compared with theoretical predictions. As models of unsaturated magnetization microparticles, MM of diamagnetic polystyrene microspheres (PSMs) in paramagnetic gadolinium solutions, and MM of spores of a bacterium containing paramagnetic manganese, *Bacillus globigii*, in diamagnetic PBS solution were measured. The superparamagnetic iron oxide nanoparticle (SPION)-labeled PSMs were selected as a model of saturated magnetization microparticles. The magnetic susceptibility of PSMs and the saturation magnetization of the SPIONs were determined by applying the simultaneous measurement capacity of CTV on MM and sedimentation velocity.

Chapter 4 describes a fluorescent version of the CTV system (Fl CTV). The sensitivity and specificity of commercially available magnetic colloids were evaluated by the combination of Fl CTV and visible version of CTV (VIS CTV).

In Chapter 5, post differentiation isolation of RBCs from hematopoietic stem cell (HSC) bioreactor cultures is described. This was performed by exploiting the
paramagnetic property of hemoglobin. The separation performance and the characterization of purified RBCs in terms of size, magnetophoresis, morphology, and deformability were evaluated by CTV, differential interference contrast (DIC) microscopy, and ektacytometry. The Hb content in individual isolated RBC was quantified.

In Chapter 6, a label-less magnetic separation method for selection of non-apoptotic human spermatozoa for potential, future application to assisted fertilization was proposed and studied. Meanwhile, the immunomagnetic cell separation method was investigated, where apoptotic spermatozoa were selectively labeled with annexin V magnetic beads and depleted using a MACS separation system. Comparisons were conducted on the separated fractions obtained from these two methods.

Chapter 7 presents a preliminary study on the intrinsic magnetophoresis of cancer cell lines. MMs of eight cancer cell lines cultivated in the complete media were measured and compared. The effects of two iron compounds, ferric nitrate and ferric ammonium citrate (FAC), introduced to the culture media, on cancer cell magnetophoresis and TfR1 expression were investigated. The label-less MACS separation was carried out to select cancer cells with potential of high intrinsic magnetic susceptibility. Cell cycle analysis on the separated and unseparated cells was further performed and compared.

Chapter 8 summarizes the conclusions and presents suggestions for future work.
CHAPTER 2

MEASUREMENT ERRORS OF CTV SYSTEM

2.1 Introduction

Cell tracking velocimetry (CTV) is designed and developed for the experimental measurement of motion of micro-sized cells or particles suspended in viscous media. Past studies in the laboratory of Dr. Chalmers at the OSU and Dr. Zborowski at Cleveland Clinic have shown a good agreement between the measured and expected particle velocity induced by the magnetic field or by gravity (sedimentation) based on the CTV measurements and known particle and fluid properties (such as particle hydrodynamic diameter, magnetic susceptibility, density, and fluid viscosity) (Chalmers et al. 1999a; Chalmers et al. 1999b; Moore et al. 2004; Zborowski et al. 2003; Zhang et al. 2005). The technique is promising, with potential applications to measuring physical properties (e.g. magnetic susceptibility) of a single cell. However, as with any analytical instrument, every CTV measurement has a certain error associated with it. In some cases, the error may be so small that it can be neglected. But in other cases, the possibility of this error cannot be overlooked. Especially when studying very weak effect, such as particle
Brownian motion or intrinsic cell magnetophoresis, it is necessary to evaluate the measurement error. In this study, the one-dimension Brownian motion of monodisperse microsphere standard is used as an experimental model to test the CTV measurement error. The measurement variance contributed by the particle size variance is so small that it could be neglected as these particles are highly monodisperse. Therefore, the measurement variance represents the CTV instrument error.

2.2 Theory

2.2.1 The displacement distribution of N Brownian particles

Figure 2.1 Particle displacements starting from $x = 0$ over a given period from 0 to $t$

Here only a one-dimension Brownian motion is discussed. This is justified by the one-dimensional particle motion analysis capability of the CTV apparatus. The
displacement, \( x \), of each particle induced by Brownian motion over same period \( t \) is different and independent, Figure 2.1. The number concentration of particles, \( c(x, t) \), is a function of position and time.

The diffusion of Brownian particles in a static fluid is described by second Fick’s law (Einstein and Fürth 1956)

\[
\frac{\partial c(x, t)}{\partial t} = D \frac{\partial^2 c(x, t)}{\partial x^2}
\]

(2.1)

where \( D \) is the particle diffusion coefficient in the fluid. Assume a collection of non-interacting particles, concentrated at a point of \( x_0 = 0 \) at \( t = 0 \), with the known total number of particles (such as \( N \), Figure 2.1). Further, assume the initial condition \( c(x, 0) = N \delta(0) \), where \( \delta(x) \) is the Dirac’s delta. Consequently, the solution of the second Fick’s equation with the initial condition as above describes evolution of the concentration profile of a collection of particles with time, \( c(x, t) \). The solution of eqn (2.1) with the total number of particles normalized to 1 (that is, \( N = 1 \)) is as follows (Bird et al. 2002):

\[
c(x, t) = \frac{1}{\sqrt{4\pi Dt}} \exp\left(-\frac{x^2}{4Dt}\right), \quad \int_{-\infty}^{\infty} c(x, t) dx = 1
\]

(2.2)

The solution is a Gaussian function with the mean \( \mu_x = 0 \) and the variance \( \sigma_x^2 = 2Dt \) and so can be re-written in a more familiar form as:

\[
c(x, t) = \frac{1}{\sqrt{2\pi\sigma_x^2}} \exp\left(-\frac{(x - \mu_x)^2}{2\sigma_x^2}\right)
\]

(2.3)

Mathematically, eqn (2.2) also represents the probability density function of the particle displacement distribution, which is a Gauss distribution. From the above it follows that the Brownian particle dispersion increases as the square root of time:
\[ \sigma_t = \sqrt{2Dt}, \quad \sigma_t \propto \sqrt{t} \] (2.4)

The diffusion coefficient of Brownian particles, \( D \), is given by Stokes-Einstein equation (Einstein and Fürth 1956)

\[ D = \frac{k_B T}{3\pi \eta d} \] (2.5)

where \( k_B \) is Boltzmann’s constant (= 1.381 \times 10^{-23} \text{ J/K} ), \( T \) is the temperature, \( \eta \) is the fluid viscosity, and \( d \) is the particle diameter.

### 2.2.2 Measurement error

Suppose the measured particle displacement, \( x \), is subject to random measurement error, \( \varepsilon \), which has a mean 0 and variance \( \sigma_\varepsilon \). Then the observed \( x' \) could be expressed as

\[ x' = x + \varepsilon \] (2.6)

where \( x \) is the “true” value of the variable \( x' \). The mean of true particle displacement at a time \( t \), \( \mu_x \), is the mean over distances available to a Brownian particle undergoing random thermal motion. And therefore it is 0 as predicted by eqn (2.2) or calculated as a mean of \( x \) with respect to a probability density function \( f(x) = \frac{c(x, t)}{\int c(x, t) \, dx} \), so that

\[ \mu_x = \frac{\int_{-\infty}^{\infty} xc(x, t) dx}{\int_{-\infty}^{\infty} c(x, t) dx} = \int_{-\infty}^{\infty} xc(x, t) dx \equiv E[x] = 0 \] (2.7)

where \( E[x] \) is the expected value of a continuous variable \( x \) distributed with the probability density function \( c(x, t) \). The last equation follows from the fact that \( c(x, t) \) is symmetric (is the Gaussian density distribution function, eqns (2.2) and (2.3)).
expected value of a random error of measurement distributed normally is also equal to zero

\[ E[\varepsilon] = 0 \]  \hspace{1cm} (2.8)

Therefore, the expected value, or the mean, of the experimentally measured value of the particle displacement, \( x' \), is also zero

\[ \mu_x = E[x'] = E[x + \varepsilon] = E[x] + E[\varepsilon] = 0 \]  \hspace{1cm} (2.9)

The observed variance is

\[ \sigma_x^2 = E[x^2] + E[\varepsilon^2] = \sigma_x^2 + \sigma_\varepsilon^2 \]  \hspace{1cm} (2.10)

where \( \sigma_x^2 = 2Dt \), eqn (2.4), and the value of \( D \) can be calculated from the known properties of the particle and the suspending fluid, eqn (2.5).

In the final analysis, the variance contributed by the instrument error, \( \sigma_\varepsilon \), is estimated as a difference between the variance of the measured particle displacement, \( \sigma_{x'}^2 \), and the variance due to the random motion of the Brownian particles

\[ \sigma_\varepsilon^2 = \sigma_{x'}^2 - \sigma_x^2 = \sigma_{x'}^2 - 2Dt \]  \hspace{1cm} (2.11)

\subsection*{2.3 Materials and methods}

\subsubsection*{2.3.1 Image recording system}

The CTV system was modified to record and measure the particles’ Brownian motion. Briefly, the permanent magnet assembly was removed from the CTV system. The other parts of the CTV system including the Olympus microscope and monochrome CCD camera were kept unchanged. A borosilicate glass channel supported by two aluminum plates was placed in front of the microscope objective. Cells’ two dimensional
motions (horizontal and vertical) were captured. Only unbiased random motions in horizontal direction were considered in this study. The motion in vertical direction was biased by gravity.

2.3.2 Monodispersed microsphere

Monodispersed microsphere size standard (Cat. No. 4009A) from Duke Scientific Corporation (Now Thermo Fisher Scientific Inc., Waltham, MA), with a mean size of 0.994 µm and standard deviation of 0.021 µm was used for the Brownian motion detection. It is traceable to the Standard Meter through the National Institute of Standards and Technology (NIST). This particle is made of polystyrene (PS) with a density of 1.05 g/cm³. Particles were diluted with distilled water before the motion tracking experiment.

2.4 Results and discussion

The discussion in Theory section shows that starting from \( x = 0, t = 0 \) over a given time \( t \), the distribution of displacement \( x(t) \) of \( N \) particles is normal with a mean of 0 and standard deviation of \( (2Dt)^{0.5} \). For the monodispersed microspheres used in this study which were suspended in water with a viscosity of \( 8.9 \times 10^{-4} \) kg/m-s, its diffusion coefficient at room temperature (298 K) is \( D = 4.94 \times 10^{-13} \) m²/s. The mean and standard deviation of the displacement over \( t = 2 \) seconds are then predicted to be 0 and \( 1.41 \times 10^{-6} \) m. Figure 2.2 presents the examples of the expected or true displacement and measured displacement distribution over different time periods. The mean and standard deviation data is shown in Table 2.1. The mean value of the displacements measured by CTV system is very close to 0. And the measured standard deviations are smaller than the
expected values for both $t = 2s$ and $t = 4s$. The gravity effect may account for it, which may weaken the randomness of Brownian motion. Moreover, the proportional relationship in eqn (2.4) was confirmed by CTV measurement. The measured standard deviation at $t = 4s$ is about $1.4 = \sqrt{2}$ times bigger than that at $t = 2s$.

$$\frac{\sigma_x(t_2)}{\sigma_x(t_1)} \approx \sqrt{\frac{t_2}{t_1}} = \frac{\sigma_x(t_2)}{\sigma_x(t_1)} \quad (2.12)$$

These results indicate that the contribution of the instrument error of CTV system to the total error of measurement is negligible for microparticles in the micrometer size range used in this study.

<table>
<thead>
<tr>
<th>$t$ (s)</th>
<th>$x$ (m)</th>
<th>$\sigma_x$ (m)</th>
<th>$x'$ (m)</th>
<th>$\sigma_x'$ (m)</th>
<th>particle #</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0</td>
<td>$1.41 \times 10^{-6}$</td>
<td>$-2.36 \times 10^{-7}$</td>
<td>$1.20 \times 10^{-6}$</td>
<td>2,545</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>$1.41 \times 10^{-6}$</td>
<td>$-1.23 \times 10^{-7}$</td>
<td>$1.29 \times 10^{-6}$</td>
<td>1,622</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>$1.99 \times 10^{-6}$</td>
<td>$-6.25 \times 10^{-7}$</td>
<td>$1.70 \times 10^{-6}$</td>
<td>1,466</td>
</tr>
</tbody>
</table>

Table 2.1 Mean and standard deviation of the “true” and measured displacement
Figure 2.2  The displacement distribution of monodispersed Brownian particles over 2s (A) and 4s (B)
2.5 Conclusions

CTV was originally developed to measure the magnetically induced motion and sedimentation of micro-sized cells or particles. It consists of several key components: (1) a well-characterized magnetic field energy density gradient; (2) a microscopic image acquisition system; and (3) a computer algorithm to determine the location and velocity of each cell or particle in the region of image analysis. In current study, the latter two in current CTV system was tested by removing the permanent magnet assembly. The sensitivity of the CTV instrument to detect particles with size as low as one micron and very weak random motion was confirmed. The experimental results agree with the theoretical prediction very well, which implies the high accuracy of our CTV system and negligible instrument error contribution to the overall error of measurement.
CHAPTER 3

DIFFERENCES IN MAGNETICALLY INDUCED MOTION OF DIAMAGNETIC, PARAMAGNETIC, AND SUPERPARAMAGNETIC MICROPARTICLES DETECTED BY CTV

3.1 Introduction

Immunomagnetic cell separation has been widely used in separating a large variety of cell types (Jing et al. 2007; Safarik and Safarikova 1999). It relies on selective attachment of magnetizable nano- or micro-sized particles to cells to produce a difference in magnetic susceptibility between different cell subsets (Hackett and St Pierre 2005; Häfeli et al. 2005; Han and Frazier 2004; Pamme 2007; Wilhelm et al. 2002; Winoto-Morbach et al. 1995). Further improvement in the performance of magnetic cell separation process depends on the development of highly specific and sensitive magnetic labels and efficient, high-throughput magnetic cell separators. Therefore, characterization of the magnetic particles themselves and the cell/magnetic label particle complex plays
an important role in the evaluation and subsequent improvement in the performance of magnetic cell separation systems.

Magnetophoretic mobility (MM) is a parameter used to measure the response of microparticulate matter suspended in a viscous fluid to the applied magnetic field (Zborowski et al. 2002). A cell labeled with magnetic nanoparticles acquires a MM that is quantitatively related to the physio-chemical properties of the cell-label complex and the suspending fluid, such as the magnetic susceptibility of the nanoparticles, the hydrodynamic diameter of the cell-label complex, the antibody binding capacity of the cell, and the viscosity of the fluid medium (McCloskey et al. 2003a). The MM distribution in the cell population depends on the selectivity and specificity of the targeting antibodies and the cell surface antigen expression (Leigh et al. 2005; McCloskey et al. 2000).

To experimentally measure the MM of single, micron-sized particles and cells, we have developed an experimental instrument, referred to as Cell Tracking Velocimetry (CTV) (Chalmers et al. 1999b; Chalmers et al. 1999c). Compared to other methods, such as Gouy and Faraday balances and a superconducting quantum interference device, SQUID, that produce bulk averages (Cantor et al. 1993; Cullity 1972), CTV measures the magnetic property of a single microparticle or a cell. The magnetically induced velocity, \( u_m \), of a cell in a constant magnetostatic energy density gradient, \( S_m \), is measured using microscope and computer tracking velocimetry software on a large set of cells, typically numbering a few thousands (Nakamura et al. 2001). Such analyses allow further improvements in both labels, labeling methodology, and cell separation instrument design and operation (Chosy et al. 2003; Jing et al. 2007; Williams et al. 1999).
In principle, the magnetic properties of materials, whether they are diamagnetic, paramagnetic, ferromagnetic, or superparamagnetic, introduce different functional dependence of MM on the applied field. In particular, the MM of particulate matter suspended in a continuous, viscous medium is directly proportional to the difference of magnetic susceptibilities between the suspended and the suspending phases, $\chi_p - \chi_f$ (particle and fluid, respectively, discussed in the next section). Therefore, the experimentally detected changes of the particle mobility with the applied field correspond to the particle (and fluid) susceptibility changes with the field. For the types of fluids used for cell separation (aqueous solutions of Na\(^+\), Cl\(^-\), phosphates, amino acids, glucose, and proteins, such as 5% w/v bovine serum albumin) the solution is diamagnetic, and therefore, the magnetic susceptibility of the liquid phase is independent of the applied field. The susceptibility of the cell-label complex, however, is largely determined by the susceptibility of the magnetic label, because the cell susceptibility is small by comparison. Typically, the microparticle magnetization saturates in the applied field and therefore, the magnetic susceptibility of the cell-label complex becomes a function of the applied field.

The high sensitivity of the CTV apparatus to the motion of single microparticles in response to the applied magnetic field, and its ability to measure mobility distribution on large sets of microparticles, allowed us to compare the functional dependence of MM on the applied field for different types of magnetic microparticles. As a model of the saturated magnetization microparticle, we have selected monodisperse, polystyrene microspheres (PSMs) complexated with dextran nanoparticles doped with magnetite. As a model of the un-saturated magnetization (paramagnetic) microparticles, the spores of a bacterium containing paramagnetic manganese, *Bacillus globigii*, were selected. Also, the
effect of a paramagnetic gadolinium solution on the mobility of unlabeled (diamagnetic) PSMs was investigated. In this study, the two different magnetic responses of the viscous suspensions of the particulate matter to varying magnetic fields are presented and discussed by using the latest version of the CTV that incorporates control over the (variable) applied magnetic field.

3.2 Theory

3.2.1 Magnetophoretic mobility

Magnetophoretic mobility, $m$, has been previously defined as the magnetically induced velocity, $u_m$, divided by the local magnetostatic energy density gradient, $S_m$:

$$m = \frac{u_m}{S_m}$$  \hspace{1cm} (3.1)

For particle motion in one dimension, applicable to the CTV magnetic field design, the parameter $S_m$ becomes:

$$S_m \equiv \frac{d}{dx} \left( \frac{B_0^2}{2\mu_0} \right) = \frac{1}{2\mu_0} \frac{dB_0^2}{dx} = \frac{1}{2} \mu_0 \frac{dH^2}{dx}$$  \hspace{1cm} (3.2)

where $B_0 = \mu_0 H$ is the magnetic field induction (in units of tesla, T), $H$ is the strength of the applied magnetic field (in units of ampere/meter, or A/m) and $\mu_0$ is the magnetic permeability of a vacuum with a value of $4\pi \times 10^{-7}$ Tm/A (S.I. units system is used throughout this work) (Becker 1982). In this paper, the applied field strength $H$ is used interchangeably with the field induction $B_0$ as the type of the media used for cell separation have negligible effect on the local field induction $B$ (Furlani et al. 2007).
In the limiting case of a microsphere undergoing creeping flow in viscous media, the MM becomes:

\[ m_{\mu s} = \frac{u_m}{S_m} = \frac{(\chi_{\mu s} - \chi_f)V_{\mu s}}{3\pi D_{\mu s} \eta} = \frac{\Delta \chi D_{\mu s}^2}{18 \eta} \]  

(3.3)

where \( D_{\mu s} \) is the diameter of the microsphere with a magnetic susceptibility of \( \chi_{\mu s} \), \( \chi_f \) is the susceptibility of the fluid, \( V_{\mu s} \) is the volume of the microsphere, and \( \eta \) is the viscosity of the fluid.

The MM of the microsphere is directly proportional to the difference of magnetic susceptibilities of the particle and the suspending media, eqn (3.3). The form of eqn (3.3) is analogous to that for the particle sedimentation coefficient (with the particle and fluid magnetic susceptibilities, \( \chi_{\mu s} \) and \( \chi_f \), playing the role of the particle and fluid mass densities, \( \rho_{\mu s} \) and \( \rho_f \), compare with eqn (3.10), below). Therefore, it has been occasionally referred to in the literature as the “magnetic Archimedes effect” (Jones and O’Grady 2005; Kitazawa et al. 2001). Consequently, the MM of a particle is a simple function of the material properties of the particle and the suspending media (in particular, their magnetic susceptibilities). When measured experimentally by CTV in media of known susceptibility and viscosity (such as aqueous solutions), MM provides a direct measure of the particle magnetic susceptibility (Moore et al. 2004; Pankhurst et al. 2003; Zhang et al. 2005). Conversely, when CTV is used in combination with calibration particle standards, such as monodisperse PSMs, the MM of such standards provides a direct measure of the unknown fluid magnetic susceptibility (Moore et al. 2004). It has been shown recently that the application of MM determination by CTV can be extended to measurements of magnetic properties of the nanoparticles themselves (Zhang et al. 2005). For the case of
the microsphere complexated with the magnetic nanoparticles attached to its surface, the magnetophoretic mobility \( m \) of the thus labeled microsphere is expressed by adding a correction \( m_{ns} \) to the mobility of the un-labeled microsphere \( m_{\mu s} \):

\[
m = m_{\mu s} + m_{ns} = \frac{(\chi_{\mu s} - \chi_j) V_{\mu s}}{3\pi D_t \eta} + \frac{N_{ns}(\chi_{ns} - \chi_j)V_{ns}}{3\pi D_t \eta}
\]

(3.4)

where \( N_{ns} \) is the number of magnetic nanoparticles bound to the microsphere, \( \chi_{ns} \) is the magnetic susceptibility of the nanoparticle with a volume of \( V_{ns} \), and \( D_t \) is the equivalent spherical diameter of the microsphere-nanoparticle complex. For the case of a cell labeled with magnetic nanoparticles, the same formula applies with the \( m_{\mu s} \) and \( D_t \) substituted by the characteristic MM and the hydrodynamic diameter of the labeled cell, respectively.

### 3.2.2 The dependence of the magnetophoretic mobility on the applied field, \( H \)

For diamagnetic and paramagnetic particles, whose magnetization, \( M \), is directly proportional to the applied field, \( H \), the magnetic susceptibility, \( \chi \), is independent of the applied field:

\[
\chi = \frac{M}{H}
\]

(3.5)

The same applies to the diamagnetic and paramagnetic media, and therefore, for such systems, the MM of suspended particles is independent of the applied field, eqns (3.3) and (3.4).

However, complications arise in CTV measurements of non-linear magnetic materials for which the ratio of particle magnetization to the applied field in eqn (3.5) is
not constant. The difficulty stems from the fact that the driving force of the magnetically induced velocity is the gradient of the magnetostatic energy density, $S_m$ (eqn (3.2)), which requires that the field, $H$, changes along the particle trajectory. As the field changes, so does the particle magnetic susceptibility, $\chi_p$. This introduces field-dependent contributions to the expression for the particle MM, eqn (3.3) which, in principle, could be measured by the CTV.

An important class of non-linear magnetic materials includes the ferromagnetic materials, such as iron oxides, which are typically contained within the nanoparticles used for magnetic cell labeling (Pankhurst et al. 2003). Another important class of material used corresponds to the superparamagnetic materials, such as superparamagnetic iron oxide nanoparticles (SPIONs) (Bean and Livingstone 1959; Kantor et al. 1998). In this case, the particle size is equal to or smaller than the theoretical size of single domain; thus, there is no domain wall movement upon magnetization. There is no hysteresis, implying, no coercivity or remnant magnetization (Jakubovics 1994). However, such particles undergo saturation magnetization at relatively weak fields, much lower than that typically used for CTV analysis (Figure 3.1). In order to extend the application of CTV analysis to ferromagnetic and superparamagnetic particles, the expression for MM of such particles has to be reinterpreted by introducing terms characteristic of non-linear magnetic materials, such as the saturation magnetization, $M_s$. This is accomplished by extending the definition of the magnetic susceptibility, eqn (3.5), to the magnetically saturated materials (Bozorth 1968). For those materials the magnetic susceptibility becomes inversely proportional to the field, $H$ (Figure 3.1):
Figure 3.1  Magnetization curves for monodisperse magnetic microspheres (adapted from reference (Moore et al. 2000)). Measurements were made using Oxford vibrating sample magnetometer. Note the dependence of particle susceptibility on field strength for fields higher than the saturation field. The saturation field is $\sim 1,000$ oersted (Oe) in CGS units system. Multiply CGS unit for $H$ (Oe) by $1000/4\pi$ to obtain $H$ (A/m) in SI unit; and multiply CGS unit for volume magnetization, $M$ (emu/cm$^3$) by 1000 to obtain $M$ (A/m) in SI unit. Volume magnetization is calculated from the mass magnetization providing that the density of the beads is known.

Furthermore, by treating the parameter $\chi$ formally, as defined by eqn (3.6), one may substitute $\chi$ in eqn (3.4) by the RHS of eqn (3.6) and thus arrive at the expression for

\[
\chi = \chi(H) = \frac{M_s}{H}
\]  

(3.6)
the MM of a PSM complexated with superparamagnetic nanoparticles in a saturating, magnetic field, as obtained by Zhang et al. (Zhang et al. 2005):

\[ m = m_{\mu s} + \frac{N_m (\mu_0 M_s - \chi_f) V_{ms}}{3\pi D\eta} \]

where \( M_s \) is the saturation magnetization of the nanoparticles and \( B_0 = \mu_0 H \). Note that the suspending fluid (physiologic electrolyte solution in water) and the PSM that binds the magnetic nanoparticles, are diamagnetic and therefore, \( \chi_f \) and \( \chi_{\mu s} \) are independent of the applied field. This equation shows that for saturated SPIONs binding to the PSM, the MM of the PSM-SPION complex decreases with the increasing, applied magnetic field, \( B_0 \).

The above analysis shows that the magnetically induced motion of the microparticles depends on the material property of the microparticle. For paramagnetic and diamagnetic particles in paramagnetic or diamagnetic fluid media (linearly polarizable materials, for short), the magnetically induced particle velocity is directly proportional to the gradient of the square of the local magnetic field intensity, \( dB_0^2/dx \). This is shown by combining eqns (3.1), (3.2) and (3.3), from which one obtains:

\[ u_m = mS_m = \frac{\Delta \chi D_{\mu s}^2}{36 \mu_0 \eta} \frac{dB_0^2}{dx}, \quad \Delta \chi = \text{const} \]

In comparison, the presence of the bound superparamagnetic nanoparticles on the surface of the PSMs introduces saturation magnetization effects that weaken the dependence of the PSM velocity on the applied field so that the velocity becomes directly proportional to \( dB_0/dx \). This is shown by combining eqns (3.1), (3.2), and (3.7), and by dropping in eqn (3.7) terms related to the diamagnetic properties of the microsphere and
the suspending fluid ($m_{\mu S}$ and $\chi_f$) that are small compared to the term related to the superparamagnetic properties of the nanoparticles ($M_s$):

$$u_m = mS_m = \frac{\mu_0 M_s N_{ns} V_{ns} dB_0}{6 \mu_0 \pi \eta D \eta} \frac{dB_0}{dx} = \frac{M_s N_{ns} V_{ns} dB_0}{3 \pi \eta D \eta} \frac{dx}{dx}, \quad M_s = \text{const} \quad (3.9)$$

### 3.2.3 The ratio of particle magnetically-induced particle velocity, $u_m$, to its sedimentation velocity, $u_g$

The direction of the magnetic field gradient in the CTV apparatus is along $x$ and orthogonal to the direction of the gravity (see Figure 3.2 (A)). Typically, the magnetically-induced velocity is on the order of magnitude of the particle sedimentation velocity, $u_g$. This provides an opportunity to normalize the particle magnetophoresis by the gravitational settling effects and eliminate parameters related to viscous drag, $\eta$ and $D_{\mu S}$. The particle sedimentation coefficient, $s$, is defined as:

$$s = \frac{u_s}{g} = \frac{(\rho_{\mu S} - \rho_f)V_{\mu S}}{3\pi D_{\mu S} \eta} = \frac{\Delta \rho D_{\mu S}^2}{18\eta} \quad (3.10)$$

where $g = 9.81 \text{ m/s}^2$ is the standard gravitational acceleration. The formal resemblance of the expression for $s$ to that of the magnetophoretic mobility, $m$ (eqn (3.3)) was already noted, above.
Figure 3.2  (A) Schematic diagram of the relative position of the electric coils and analysis channel for the electromagnetic CTV system. 1, 2 — pole pieces and flux return yolk made of 1018 low-carbon steel; 3 — copper wire coil. (B) Example of the computer screen output of the CTV software indicating the settling trajectories (vertical traces) and magnetically induced trajectories (horizontal traces) of particles in ROI tracked by the CTV system.

Continued
3.2.4 Linearly polarizable magnetic materials, $\Delta \chi = \text{const.}$

Dividing eqn (3.3) by eqn (3.10), one eliminates $\eta$ and $D_{\mu s}$ to obtain:

$$\frac{u_m}{u_g} = \frac{(\chi_{\mu s} - \chi_f) S_m}{(\rho_{\mu s} - \rho_f) g}$$

(3.11)

Eqn (3.11) can be further rearranged so that:

$$\frac{u_m}{u_g} \frac{g \Delta \rho}{S_m} = \chi_{\mu s} - \chi_f$$

(3.12)

Therefore, a plot of $u_m g \Delta \rho / u_g S_m$ as a function of $\chi_f$ is expected to provide a straight line in which the $y$-intercept is the magnetic susceptibility of the microsphere.

3.2.5 Magnetically saturated materials, $M_s = \text{const.}$

Dividing eqn (3.9) by eqn (3.10), in which $D_{\mu s}$ is substituted by $D_t$, one arrives at:
where $M_{s,\text{ave}}$ is the weighted average saturation magnetization of the PSM complexated with superparamagnetic nanoparticles, which has a total volume of $V_i$:

\[
M_{s,\text{ave}} = M_s \frac{N_{ms} V_{ms}}{V_i} = \frac{6M_s N_{ms} V_{ms}}{\pi D_i^3}
\]  

(3.14)

Here, a plot of $u_m/u_g$ against $dB_0/dx$ is expected to produce a straight line. By measuring the ratio of the magnetically induced velocity and the settling velocity at different values of the magnetic field gradient, and reducing the data to the plot of $u_m/u_g$ as a function of $dB_0/dx$, one obtains the saturation magnetization of the labeled microsphere complex, $M_{s,\text{ave}}$, from the slope.

In summary, the theoretical analysis of the particle magnetophoresis in a well defined magnetic field leads to quantitative predictions of material properties of the particle (its magnetic susceptibility and density) and the effect of superparamagnetic nanoparticle binding (average saturation magnetization of the microsphere-nanoparticle complex). In this study, these predictions are tested by using the latest version of the CTV system in which the permanent magnet assembly has been replaced with electromagnets allowing control over the magnetophoretic driving force, $S_m$.

### 3.3 Materials and methods

#### 3.3.1 Variable-field version of CTV

To increase the capability of CTV to analyze magnetic particles and cells over a range of magnetic energy density gradients, $S_m$, the permanent magnet assembly was
replaced with an electromagnetic system. Each electromagnet consisted of a coil surrounding a central pole piece which replaces the permanent magnets. The core was constructed of 1018 low-carbon steel and the coil was wound with 1900 turns of 18 American Wire Gage (AWG) copper wire. The electromagnet was designed to have a magnetic energy density gradient that could range from zero to a maximum of 106 TA/mm², approximately equivalent to the previously described permanent magnet, CTV system (Chalmers et al. 1999c). These electromagnets were then placed in the previously designed CTV magnetic circuit such that the interpolar gap which produced the magnetic energy density gradient was unchanged. Figure 3.2 (A) is a diagram of the coils and the magnetic circuit. The current to the coils is supplied by a programmable DC power supply (Model HPD 60-5, Xantrex, Vancouver, British Columbia). It is operated in a constant current mode and has a range of 0 to 5 A. In the constant current mode, an internal feedback control loop adjusts the voltage to keep a constant current if the resistance of the coils changes. The power supply is interfaced with the computer using a GPIB connection. It is controlled using the CTV software. The dependence of the magnetic field \( B_0 \) and the magnetic energy density gradient, \( S_m \), on the electric current, \( I \), at the center of the microscope’s field of view is shown in Figure 3.3.

The microparticle suspension to be analyzed is pumped into a rectangular borosilicate glass channel which is placed in the interpolar gap of the electromagnet as shown in Figure 3.2 (A). The microparticle motion analysis is performed in the stationary fluid (no convective flow) after two valves located at the ends of the channel are closed. The motion of microparticles induced by gravity or magnetic force in the region of interest (ROI) is recorded using an inverted microscope and a 30Hz Cohu CCD 4915
camera (Cohu Electronics, San Diego, CA). The captured images are processed by CTV software, which produces an Excel™ (Microsoft Corp., Redmond, WA) file with microparticle positions for 20 equal time intervals, used to calculate microparticle velocity and sample statistics. A computer screen image after the CTV program has tracked particle settling trajectories (in the vertical direction) and the trajectories for the same particles after the magnetic field was switched on (in the horizontal direction) is shown in Figure 3.2 (B). Additional details of the hardware and software components of the CTV system are described in the Appendix A.

![Graph showing magnetic energy density gradient, $S_m$, and the applied magnetic field, $B_0$, as a function of electric current in coils at the center of microscope field of view](image)

Figure 3.3  Magnetic energy density gradient, $S_m$, and the applied magnetic field, $B_0$, as a function of electric current in coils at the center of microscope field of view
3.3.2 Solution susceptibility modifiers

The magnetic susceptibility of the suspending fluid was adjusted with a chelating agent and paramagnetic ion, gadolinium, Gd$^{3+}$, which is marketed under the brand name Optimark® (Mallinckrodt Inc, St. Louis, MO) (Moore et al. 2004). A phosphate buffered 150 mM saline (PBS) solution was used as a reference ($\chi_f \approx -9.05 \times 10^{-6}$).

3.3.3 Linearly polarizable magnetic microparticles – *Bacillus globigii* spores

Melnik et al. recently reported the observation that the spores of at least three strains of *Bacillus*: *Bacillus atrophaeus* (formally *Bacillus globigii*), *Bacillus thuringiensis*, and *Bacillus cereus* demonstrated significant intrinsic magnetic susceptibility (Melnik et al. 2007). All three strains when sporulated demonstrated significant MM using the CTV system. Energy dispersive spectroscopy confirmed that this magnetic susceptibility is the result of the presence of the paramagnetic element, manganese (Mn). The *B. globigii* spore suspensions were prepared as described in the original article (Melnik et al. 2007).

3.3.4 Magnetically saturated materials - micro and nanoparticles

Biotinylated PSMs, SPHERO™ Biotin Polystyrene Particles (Cat. No.TP-60-5, Lot number v01, Spherotech Inc., Libertyville, IL) were used in this study. The data sheet for the particles provided by the manufacturer reports that the mean size, based on SEM analysis, is 6.7 micron.

Four types of magnetic nano- and micro-particles were used in this study: MACSTM Anti-Biotin Microbeads (Cat. No. 120-000-900, Miltenyi Biotec, CA, USA);
Captivate™ ferrorfluid streptavidin (Cat. No. C-21476, Molecular Probes, Eugene, OR); BD Streptavidin Imag™ particles-DM (Cat. No. 551307, BD Pharmigen, CA, USA); and Dynabeads® MyOne™ streptavidin C1 (Cat. No. 650.01, Dynal Biotech ASA, Oslo, Norway). The complexation of the PSMs with four nanoparticle preparations was performed by applying the protocol of Zhang et al. (Zhang et al. 2005). A final microsphere concentration of $5 \times 10^5$/mL was used for each CTV analysis.

3.4 Results and discussion

3.4.1 Comparison of particle settling and magnetically-induced velocities

The particle diameter calculated by the Coulter Counter method (Figure 3.4 (A)) is based on the difference in particle electrical impedance and that of the suspending media (Coulter 1956). The particle diameter can be also calculated from the particle settling velocity measured by CTV (Figure 3.4 (B)) providing that the difference between particle density and that of the suspending fluid media is known, eqn (3.10). First, we have determined the unknown particle density by applying the particle mean diameter from the Coulter Counter analysis and the mean settling velocity from CTV analysis to eqn (3.10), to obtain a mean density of 1.052 g/cm$^3$ for the PSM. This is consistent with the value of 1.05 g/cm$^3$ reported by its manufacturer. Second, the mean density of the PSM was used to calculate the PSMs diameter histogram from the bead settling velocity histogram (Figure 3.4 (A)). The two PSM diameter distribution histograms (from Coulter and CTV analyses) were compared, as shown in Figure 3.4 (C). The modes of the main peaks coincided, as expected. However, those of the minor peaks did not. This appears to
be related to a broader distribution of the CTV data than those based on the Coulter analysis.

Figure 3.4  (A) Distribution of PSM diameters based on Coulter Counter analysis. (B) The PSM settling velocity, $u_g$, measured by CTV. (C) Superposition of Coulter Counter histogram and the diameter of the PSMs calculated from CTV settling velocity distribution shown in (B) using an average PSM density of 1.052 g/cm$^3$. 

55
Figure 3.5 A plot of the diameter of unlabeled and labeled PSMs using a density of 1.052 g/cm³ for both populations of spheres, (A). The labeled PSMs were labeled with MACSTM anti-biotin nanoparticles. (B) is a dot plot of the MM and diameter of the unlabeled and labeled PSMs presented in (A). Note shift in the PSMs mobility and the apparent diameter upon binding of the magnetic nanoparticles.

The effect of magnetic nanoparticle binding on the settling velocity of the PSM-nanoparticle complex is illustrated by a shift in the apparent PSM diameter measured by the CTV. The same PSM density of 1.052 g/cm³ was used to calculate the diameter of the PSM-nanoparticle complex. Figure 3.5 (A) shows the histograms of the diameter distributions of the PSMs, one unlabeled and the other immunomagnetically labeled with MACSTM anti-Biotin nanoparticles. A pronounced increase in the apparent PSM diameter (from 6.53 ± 1.42 µm to 7.20 ± 0.73 µm) was observed following complexation with the nanoparticle label. The combined effect of the magnetic nanoparticle label binding to the PSM on MM and on the settling velocity was shown in a dot plot, with the apparent
particle diameter shown on the horizontal axis and the particle MM on the vertical axis, Figure 3.5 (B). A clear separation of the two sets of data (unlabeled and labeled PSMs) can be observed. This is interpreted as resulting from the high sensitivity of the CTV analysis to changes in the individual particle motion caused by the magnetic nanoparticle binding.

### 3.4.2 Linearly polarizable magnetic materials, $\Delta \chi = \text{const.}$

*Magnetophoretic mobility of polystyrene particles in paramagnetic solutions.*

![Graph](image)

Figure 3.6 (A) MM of unlabeled PSMs as a function of $S_m$ ranging from $10.3$ to $106$ TA/mm$^2$. The three lines correspond to the three sets of experiments conducted in solutions of different magnetic susceptibility. (B) The ratio of magnetically induced velocity to settling velocity as a function of magnetic susceptibility of the suspending buffer. Note: the error bar represents the 95% confidence interval of the mean value.
The theory predicts that the MM of the PSMs is independent of $S_m$, eqn (3.3). This was tested in a set of experiments in which unlabeled PSMs were suspended in solutions of paramagnetic gadolinium at three different concentrations: 0.0625, 0.1, and 0.1667 mol/L, which corresponds to a $\chi_f$ of $1.22 \times 10^{-5}$, $2.49 \times 10^{-5}$, and $4.76 \times 10^{-5}$. The values of susceptibility were calculated from relationships reported by Zhang et al. (Zhang et al. 2005) Changes in the fluid magnetic susceptibility, $\chi_f$, cause changes in the difference between the PSM and the fluid magnetic susceptibilities, $\chi_{\mu s} - \chi_f$, and therefore are expected to lead to changes in the observed PSM magnetophoretic mobility (eqn (3.3)). This was confirmed by the CTV analysis, showing increasing magnitude of the PSM mobility with the increasing Gd concentration in solution, Figure 3.6 (A). (The negative values of the PSM mobility derive from the higher susceptibility of the fluid medium than
that of the PSM, $\chi_{\mu s} < \chi_f$.) The results also show that the PSM mobility was independent of the applied magnetic field, represented by the magnetic energy density gradient, $S_m$ ranging from 10.3 to 106 TA/mm² for each Gd³⁺ concentration, Figure 3.6 (A), as expected of the linearly polarizable magnetic materials, eqn (3.3). We have further reduced the data from Figure 3.6 (A) to a single plot using eqn (3.12), and added a datum point corresponding to PSM mobility measurement in phosphate buffered saline (PBS, $\chi_f \approx -0.90 \times 10^{-5}$), as shown in Figure 3.6 (B). Here the data points lie on a straight line, again as expected of the linearly polarizable materials (eqn (3.12)). The regression analysis confirms a high degree of correlation between $u_m g \Delta \rho / u_g S_m$ and $\chi_f$ ($R^2 = 0.996, p = 0.0013, N = 24$, slope = $-0.974 \pm 0.035$, expected -1). The y-intercept, $\chi_{\mu s}$, is equal to $-0.80 \pm 0.10 \times 10^{-5}$ and is the same (within the experimental error) as that reported by Zhang et al. ($-0.77 \times 10^{-5}$) and others (the magnetic susceptibility of polystyrene is $-0.75 \times 10^{-5}$, as quoted by CRC Handbook of Chemistry and Physics (Weast and Astle 1979) and $-0.82 \times 10^{-5}$ reported by Watarai et al. (Watarai and Namba 2001)).

**Magnetophoretic mobility of B. globigii spores**

The *B. globigii* spore suspensions in PBS are an example of paramagnetic species and, therefore, the MM of the spores is also expected to be independent of the applied field energy gradient, $S_m$ (eqn (3.3)). This was confirmed by CTV analysis, Figure 3.7. Note the large magnitude of the spore MM, larger than that measured for PSM suspensions in Gd solutions, Figure 3.6 (A). This is related to the magnetization of Mn contained in the spores. The positive value of spore MM reflects the fact that here $\chi_p > \chi_f$. A correlation analysis confirmed that the null hypothesis of the spore mobility being
independent of the applied field could not be rejected (Spearman rank order correlation coefficient = -0.267, \( p = 0.462, N = 9 \)).

Figure 3.7  MM of the *Bacillus globigii* as a function of \( S_m \) ranging from 20.2 to 142 TA/mm\(^2\). Note: the error bar represents the 95% confidence interval of the mean value.

### 3.4.3 Magnetically saturated materials, \( M_s = \text{const.} \)

*Magnetically labeled polystyrene microspheres*

In contrast to the paramagnetic species, discussed above, MM was found to be a strong function of \( S_m \) for the PSM labeled with the commercial, magnetic nano- and micro-particles. Figure 3.8 (A) is a plot of MM as a function of \( S_m \) from 0.06 to 80 TA/mm\(^2\) for the biotinylated PSM labeled with MACSTM anti-biotin nanoparticles, CaptivateTM ferrofluid streptavidin, Dynabeads® MyOne™ streptavidin, and BD™
Streptavidin Imag-DM. Once the PSMs are labeled with superparamagnetic nanoparticles, a highly non-linear dependence of $m$ on $S_m$ is observed, which is consistent with the saturation magnetization effects of the superparamagnetic compounds bound to the PSMs (eqn (3.7)). To further underscore this observation, we note that the magnetically-induced velocity, $u_m$, of the PSMs labeled with superparamagnetic nanoparticles is a linear function of the magnetic field gradient, in the saturating magnetic field (eqn (3.13)). In order to verify that prediction, we have re-plotted the data from Figure 3.8 (A) using $dB_0/dx$ as an independent variable, Figure 3.8 (B). Indeed, the linear relationships were obtained, indicating that the magnetically labeled PSMs exhibit a saturation magnetization above a $B_0$ value of approximately 0.08 T which corresponds to a $dB_0/dx$ value of approximately 0.008 T/mm.

Continued
Figure 3.8 continued

Figure 3.8 (A) MM as a function of $S_m$ for the PSMs labeled with: MACS anti-biotin nanoparticles, Captivate Ferrofluid, Dynabeads MyOne, Imag DM particles. (B) The magnetically induced velocity as a function of $dB_0/\alpha x$. (C) The ratio of the magnetically induced to the settling velocity as a function of $dB_0/\alpha x$ (only data above the saturation point, $dB_0/\alpha x \approx 0.008$ T/mm). Note: the error bar represents the 95% confidence interval of the mean value.
By plotting the ratio of magnetically-induced to settling velocity, $u_m/u_g$, of the particle as a function of $dB_0/dx$ (above the saturation point, $dB_0/dx \approx 0.008 \, \text{T/mm}$), and finding the slope of the straight line corresponding to the experimental data, one is able to calculate the average saturation magnetization, $M_{s,\text{ave}}$, of the PSM-nanoparticle complex, eqn (3.13). The results are shown in Figure 3.8 (C) for the four different combinations of PSM-magnetic nanoparticle complexes. Linear relationships were obtained for each combination, as expected for the magnetically saturated beads. Table 3.1 presents the slope, intercept, and $R^2$ for each of the four combinations. From these values, the saturation magnetization of the labeled microsphere complex, $M_{s,\text{ave}}$, were determined from eqn (3.13) and are presented in Table 3.1. Note that the magnetically induced velocity is 5 to 15 times greater than the gravitational sedimentation velocity ($5 < u_m/u_g < 15$). By combining the mean diameter data of the unlabeled and labeled PSM obtained
from CTV, the saturation magnetization of the four magnetic nanoparticles were solved from eqn (3.14) and are presented in Table 3.2.

<table>
<thead>
<tr>
<th>Type of nanoparticle</th>
<th>Slope (mm/T)</th>
<th>Intercept ($u_m/u_g$)</th>
<th>$R^2$</th>
<th>$M_{s,ave}$ (A/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MACS</td>
<td>507</td>
<td>5.0</td>
<td>0.999</td>
<td>256</td>
</tr>
<tr>
<td>Captivate</td>
<td>1,096</td>
<td>11.9</td>
<td>0.994</td>
<td>554</td>
</tr>
<tr>
<td>Dynabeads</td>
<td>491</td>
<td>14.9</td>
<td>0.969</td>
<td>248</td>
</tr>
<tr>
<td>Imag DM</td>
<td>1,068</td>
<td>7.2</td>
<td>0.997</td>
<td>540</td>
</tr>
</tbody>
</table>

Table 3.1 Numeric value of the slope, intercept, $R^2$ of the data presented in Figure 3.8 (C) and calculated saturation magnetization of the labeled PSM complex determined from eqn (3.13). The abbreviations are explained in the text accompanying eqn (3.13).

<table>
<thead>
<tr>
<th>Type of nanoparticle</th>
<th>Calculate $D_t$ (µm)</th>
<th>$V_t$ ($m^3$)</th>
<th>$N_{ns}V_{ns}$ ($=V_t-V_{µs}$, $m^3$)</th>
<th>$M_s$ (A/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MACS</td>
<td>7.20</td>
<td>$1.95 \times 10^{-16}$</td>
<td>$3.80 \times 10^{-17}$</td>
<td>1,318</td>
</tr>
<tr>
<td>Captivate</td>
<td>7.97</td>
<td>$2.65 \times 10^{-16}$</td>
<td>$1.08 \times 10^{-16}$</td>
<td>1,365</td>
</tr>
<tr>
<td>Dynabeads</td>
<td>8.19</td>
<td>$2.88 \times 10^{-16}$</td>
<td>$1.30 \times 10^{-16}$</td>
<td>548</td>
</tr>
<tr>
<td>BD Imag</td>
<td>7.78</td>
<td>$2.45 \times 10^{-16}$</td>
<td>$8.91 \times 10^{-17}$</td>
<td>1,495</td>
</tr>
</tbody>
</table>

Table 3.2 Size characteristics of the labeled PSM-nanoparticle complexes and the saturation magnetization of the magnetic nanoparticles. The abbreviations are explained in the text accompanying eqns (3.4) and (3.7)
Error Analysis

The saturation magnetization, $M_{s,ave}$ presented in Table 3.1 is an average value for the PSM-magnetic nanoparticles complex. It was calculated with the assumption that the susceptibility contribution from the microsphere and that of the solution is negligible compared to that of the nanoparticles. We estimated the error of this assumption, as well as made an estimate of the value of the $M_{sat}$ of the magnetic particles themselves. This analysis is based on the full expression for the magnetophoretic mobility, eqn (3.7). First, inspection of eqn (3.7) indicates that the term $\chi_f$ (-9.05 \times 10^{-6} for the PBS buffer) is small as compared to $\mu_0 M_s/B_0$ (which ranges from 0.003 to 0.16). To obtain this range of $\mu_0 M_s/B_0$, the range of $B_0$ in the experiments presented in Figure 3.8 (A-C) was from 0.03 to 1.03 T. Secondly, the MM of PSM, $m_{\mu s}$, in PBS solution is small (2.64 \times 10^{-6} mm^3/TAs, Figure 3.5 (B)) as compared to that of the labeled PSM (Figure 3.8 (A)), which justified its omission in eqn (3.7). Overall, the diamagnetic effects of the PSM in PBS were at least three orders of magnitude smaller than the superparamagnetic effects of the nanoparticles attached to the PSM, which justified their not being included in the expression in eqns (3.9) and (3.13).

In summary, the results show that the MM of the PSM microparticle does not depend on the applied field, as expected of the paramagnetic species. Above the saturating field value, the MM of the PSM-SPION complex decreases in inverse proportion to the applied field, as expected of the magnetically saturated species. The agreement with the theory was demonstrated by showing that for particles that do not saturate in the applied field, the magnetically induced velocity is directly proportional to
the gradient of the square of the field, $dB_0^2/dx$, and that for magnetic label nanoparticles that saturate in the applied field, the magnetically induced velocity of the PSM-label complex is directly proportional to the gradient of the magnetic field, $dB/dx$.

### 3.5 Conclusions

Cell motion analysis using the microscopic technique of cell tracking velocimetry (CTV) is, in principle, sensitive to the local stresses and the body forces acting on the cell. Our previous studies using a constant magnetic field have demonstrated quantitatively an increase of cell velocity following magnetic nanoparticle binding (Chalmers et al. (Chalmers et al. 1999c), increase of PSM velocity with a concurrent increase in magnetic susceptibility of the fluid medium (Moore et al. (Moore et al. 2004); Zhang et al. (Zhang et al. 2005)), and increase in erythrocyte velocity with conversion of intracellular iron from low-spin to high-spin state (Zborowski et al. (Zborowski et al. 2003)).

However, the use of constant magnetic field precluded us from testing if the CTV analysis is sensitive to the type of the cell magnetization (paramagnetic versus superparamagnetic), in other words, if it is capable of distinguishing between a paramagnetic and superparamagnetic response of a microscopic particle to the applied field. This has now become possible with the introduction of the variable-field CTV equipped with electromagnets and a controlled current power supply. A convenient measure of the microscopic particle response to the applied field is its magnetophoretic mobility (MM), $m$, a quantity that is directly proportional to the difference between magnetic susceptibilities of the particle and the fluid media. Thus, for a microparticle and a fluid medium whose susceptibilities are independent of the applied field (characteristic
of paramagnetic and diamagnetic materials) the microparticle MM is independent of the applied field. In comparison, the presence of the superparamagnetic species results in a decrease of microparticle MM with the increasing field. We have verified the predicted behavior of the microparticle MM in the variable magnetic field on a model of linearly polarizable (paramagnetic and diamagnetic) materials using PSMs in Gd solutions and *B. globigii* spores (known to contain paramagnetic Mn) in PBS solution, and on magnetically saturated materials using PSMs complexated with iron oxide nano- and micro-particles. As predicted, the microparticle MM was constant for linearly polarizable media and was an inverse function of the applied field for the magnetically saturated microparticles, in the range of the applied fields. In addition, the quantitative analysis of the microparticle motion by the CTV and the high statistical power afforded by the ability of multi-particle tracking per frame (producing hundreds to thousands of microparticles tracked per sample) allowed us to calculate characteristic magnetic properties of the microparticles, such as the magnetic susceptibility of the PSMs, and the average saturation magnetization of the PSM-nanoparticles complex. The accuracy of the CTV analysis was confirmed by showing that the calculated PSM susceptibility is equal to that reported by others (within experimental error) and that the PSM settling velocity is equal to the Stokes velocity predicted for the same PSM diameter and density.

The extended capabilities of the electromagnet-based CTV analysis to distinguish between the paramagnetic and superparamagnetic properties of a single microparticle or a cell will be further evaluated in future applications to cell biology, in particular, to cell pathologies.
CHAPTER 4

SPECIFICITY AND SENSITIVITY TESTING OF IMMUNOMAGNETIC LABELING REAGENT BY VISIBLE AND FLUORESCENT CTV

4.1 Introduction

The two-step labeling protocol is a widely used method in immunomagnetic cell separation and detection. Compared to a one-step labeling method, it is more complicated but preferable, when one-step magnetic labels are either unavailable or of insufficient magnetite content. The latter can give rise to magnetic moments too low for practical cell separation in the case of dimly expressed surface antigens. Typically, cells are labeled with a fluorescent primary antibody, which are then labeled with a secondary magnetic colloid tagged antibody. It allows for both magnetic separation and flow cytometry (FCM) analysis to monitor the labeling and separation outcome. Cells with magnetic particles are enriched or depleted by a MACS™ column, a magnetic flow sorter, and so on. Ideally, if both antibodies bind all the available antigens, all the target cells are both fluorescent and magnetic positive (F⁺M⁺), defined as signal strength above threshold values. However,
given the variation in the quality of current antibody reagents, the quantity of antibody reagents added, the number of antigens expressed per cell surface, cell size and age, steric exclusion, and so on, up to four subpopulations are produced after a two-step labeling procedure (Figure 4.1). In a cell mixture, it is expected that the non-target cells should be fluorescent and magnetic negative (FM⁻). In a simple system with only one cell population, because of apoptosis or other reasons, some target cells will be devoid of antibodies (FM⁻), just as control samples without labeling. However, due to the interaction of the secondary antibody with non-target surface molecules, some cells become magnetic, which are included in a non-fluorescent, magnetic subpopulation group (FM⁺). The proportion of these two subpopulations is very small if a single, only one healthy culture cell line is used for labeling. Varying amounts of these two subpopulations are generated when a mixture is used. On the other hand, without regard to the specificity and number of secondary magnetic particles incubated with the cells, a Gaussian distribution of the magnetophoretic mobility (Figure 4.2) is generated. Therefore, even as most cells are both fluorescent and magnetic (F⁺M⁺), the loading of the two antibodies varies cell by cell. Cells appearing in the extreme left side of the mobility distribution curve are fluorescent labeled, but non-magnetic (F⁺M⁻) or just FM⁻. Because of this diverse labeling, 100% recovery couldn’t be obtained even with a perfect magnetic sorter.
Figure 4.1 Four subpopulations ($F^+M^+, FM^-, F^+M^-, FM^+$) existed in one sample after a two-step labeling process.

Figure 4.2 MM histograms of two-step labeled cells at different Ab concentration (the ratio of primary and secondary Ab is 1:1)
Methods to evaluate the performance of magnetic sorters and the labeling procedure were developed. FCM is the most popular technique which evaluates the cell according to the fluorescent intensity (FI), granularity (via side scatter or SS) and size (via forward angle light scatter or FS). But it is costly and needs technical expertise in handling the equipment and interpreting data (Hausmann et al. 1998) Moreover, it is of limited worth in the design, optimization, and operation of magnetic cell separators. A more recent method developed by the author’s group is to characterize a cell by its magnetophoretic mobility (MM) and settling velocity (correlated to cell size and density). MM is a measure of the motion of a magnetically-susceptible particle in a viscous medium as a result of an applied magnetic field, which has analogies to the sedimentation coefficient \((s)\). To quantify the MM of cells, an instrument was developed and is continuing to be improved by us, which is referred to as cell tracking velocimetry (CTV) (Chalmers et al. 1999c). It measures the magnetically induced velocity, \(u_m\) of a cell, on a cell-by-cell basis, in a constant magnetostatic field energy gradient, \(S_m\). It was already demonstrated that CTV could quantify not only the antibody binding capacity of cells (McCloskey et al. 2000), but also the secondary antibody binding amplification (McCloskey et al. 2001a).

Currently, no reports of technologies are found to quantify these four subpopulations after a two-step labeling procedure. Although using CTV to measure MM was demonstrated to be a good way to assess the effectiveness of antibody-magnetic particle conjugates to label cells of interest and to help to design, predict, and optimize magnetic cell separation (Zborowski et al. 2002), the previous visible version of CTV (VIS CTV) could only distinguished the magnetically-labeled cells from non-labeled
cells. That is, only two, and sometimes three subpopulations could be detected. However, equipped with a fluorescent (Fl) microscope with light excitation and emission filters, the updated CTV system (called Fl CTV) is also capable of detecting the fluorescent signals on the cells produced by the primary labeling reagent.

In this paper, the specificity of the magnetic tagging antibody was defined as unity minus the fraction of cells labeled with the magnetic colloid alone (ideally, equal to 100%). The sensitivity of the magnetic tagging antibody was defined as the $F^+M^+$ fraction (ideally, equal to 100%). A simple system with only one Jurkat cell line was selected and labeled with a two-step labeling protocol. After quantifying the four subpopulations as a result of labeling, the specificity and sensitivity of three commercial magnetic tagging antibodies were determined. Moreover, the upgraded CTV system is used to evaluate the maximum MM of a magnetic tagging antibody without a complete titration study and combining with FCM to determine the fraction of available antigens bound by the antibody.

4.2 Theory

4.2.1 Correlation between cell FI and the cell MM

The cell fluorescence results from labeling with the monoclonal antibody-fluorochrome conjugate. In the chosen cell model and label, the intrinsic cell fluorescence is small when compared to the label fluorescence, and is ignored in subsequent analyses. In simple terms, the cell fluorescence intensity (FI) is a function of many variables, including the number of antigen targets on the cell surface ($n_1$); the fraction of the antigen targets occupied by the bound antibody ($\theta_1$); the number of targets shared by a single
antibody or, alternatively, the number of antibodies bound to a single target antigen, the antibody valence, \((\lambda_1)\); the number of the fluorochrome molecules conjugated to the antibody molecule \((n_2)\); and the fluorescence intensity of the fluorescein molecule itself \((I_0)\). Assuming that the variables are independent of each other, the resulting cell fluorescence intensity is a product of those variables:

\[
FI = f(n_1, \theta_1, \lambda_1, n_2, I_0) \approx n_1 \theta_1 \lambda_1 n_2 I_0
\]  

(4.1)

The parameter \(n_1\) is an inherent cell property and is cell type specific. Its distribution in the population results from the cell-to-cell variations in the cycle stage, the metabolic state and other factors. The parameter \(\theta_1\) is inherent of the bound antigen-free antibody chemical reaction and the antibody affinity to the antigen. The concentration of the bound antigen-antibody complex at equilibrium is determined by the mass action and is proportional to the product of concentrations of the antigen and the antibody in solution. The parameter \(\theta_1\) is a gross measure of the resulting bound antigen sites and may vary from 0 (no bound label) to 1 (saturating amount of the bound label). Typically, the cell labeling conditions are selected such that one approaches saturation of the available binding sites. This is accomplished by a titration study: incubating cells with increasing concentrations of the free antibody in solution and measuring mean cell FI in a sample by flow cytometry. The remainder of the parameters in eqn (4.1) is related to the properties of the labeling antibody, as listed above. For a well-characterized antibody-fluorochrome conjugate, the variability in the parameters \(\lambda_1, n_2, \) and \(I_0\) is much smaller than that of the antigen expression distribution in the sample; however, its contribution to the cell FI may not be entirely ignored. The determination of the exact values of those parameters is difficult. Therefore, a number of indirect measures of \(n_1\) parameter have been adopted,
such as mean equivalent standard fluorescence (MESF, equal to \( FI/I_0 \) in the notation adopted in eqn (4.1)) or the antibody binding capacity (ABC, equal to the product of \( n_1 \theta_1 \lambda_1 \)). The parameter \( n_2 \) is related to the fluorochrome-to-protein ratio, or \( F/P \) parameter, frequently used as a measure of brightness (quantum yield) of the antibody-fluorochrome conjugate. For a well-characterized cell and immunofluorescence labeling system, and for saturating concentration of the labeling antibody, the FI distribution is a facsimile of the surface antigen distribution (Figure 4.3). The cell FI distribution from asynchronic single cell culture is very nearly log-normal. This derives primarily from the variable \( n_1 \), due to the fact that cell surface area is distributed log-normally, a characteristic of growth rates in biological systems.

![Cell FI distribution](image)

Figure 4.3  Cell FI distribution (black, unlabeled, and blue, labeled with 10 µL/10⁶ cells antibodies conjugated with PE)
We have investigated if the same functional dependence holds between the magnetophoretic mobility and the cell surface antigen expression level resulting from immunomagnetic labeling. In order to maintain the one-to-one relationship between the antigen expression and the cell mobility, we have used a secondary antibody that is directed against the fluorochrome molecule conjugated to the primary antibody. The resulting cell magnetic polarization, $P$ (relative to the suspending fluid) is expected to be a function of the cell FI, and in the simplest case, a linear function of the cell FI:

$$P = f((FI / I_0), \theta_2, \lambda_2, n_3, P_0) = \frac{FI}{I_0} \theta_2 \lambda_2 n_3 P_0$$  \hspace{1cm} (4.2)$$

The parameters $\theta_2$, $\lambda_2$ and $n_3$ describe the fraction of the binding sites on the primary antibodies occupied by the secondary antibody, the number of the secondary antibodies shared by the single primary antibody, and the number of the magnetic particles attached to the secondary antibody, respectively. They are analogous to the parameters appearing in eqn (4.1). $P_0$ is the magnetic polarization of a single magnetic particle relative to the fluid:

$$P_0 \equiv \Delta \chi V_p$$ \hspace{1cm} (4.3)$$

and $\Delta \chi \equiv \chi_p - \chi_f$ is the difference in magnetic volume susceptibility between the magnetic particle and the surrounding fluid, $V_p$ is the mean particle volume. In separation science, the particle magnetic polarization plays a role of the field-particle interaction parameter, important in transport-based separations, as it gives rise to a driving force that moves the particle against the frictional forces of the viscous media. In the immunomagnetic sandwich model described by eqn (4.2), each magnetic particle bound to the cell contributes to the total cell magnetic polarization, $P$, and the intrinsic cell polarization is assumed to be small, and therefore is ignored. This holds true for magnetic particles and
suspending fluids whose magnetic susceptibility is independent of the applied field (paramagnetic substances at any field strength, and superparamagnetic or ferromagnetic substances at low field strength). Homogeneous fields are unsuitable for inducing cell motion, as both polarization and field gradient are necessary to produce a net magnetic force. (The homogenous field exerts torque but no net force). For the limited cell dimension compared with the magnitude of the field gradient used in this study, and in agreement with the limiting assumptions used for formulas in eqns (4.1) and (4.2), the polarization of the magnetic labeling particles on the cell are assumed to be the same. However, the polarization may change with position in the field, as susceptibility is not constant with superparamagnetic materials at high field strength.

In the presence of the magnetic field gradient, the cell at rest becomes mobile with the characteristic magnetophoretic mobility defined as the ratio of its magnetic polarization and the viscous friction coefficient, $f$:

$$m = \frac{P}{f} = \frac{F}{\mu_0 n_1 \lambda_2 n_3 P_0} = \frac{F}{\mu_0 n_1 \lambda_2 n_3 \Delta \chi V_0} = \frac{2 F}{\mu_0 n_1 \lambda_2 n_3 \Delta \chi R^2} \eta$$

(Eqn 4.4) describes a functional relationship between cell magnetic and fluorescence properties. We have set out to investigate them experimentally. First, we tested the assumption about the one-to-one correspondence between the cell FI and the cell magnetization. This may not be strictly true. The random nature of the antigen-antibody reaction results in a small proportion of cells binding the fluorescent antibody at below a statistical detection limit. The same applies to the secondary antibody: a small proportion of cells will bind fluorescent labels at a level below the statistical limit of detection. On the other hand, the imperfect specificity of the secondary antibody may
lead to a rare event of the magnetic particle binding to a non-fluorescent cell. The resulting combinations of cell-label complex sets are illustrated in Figure 4.1.

Second, the functional form of eqn (4.4) states a linear relationship between the cell magnetophoretic mobility (MM), $m$, and its fluorescence intensity, $F_I$. It follows, then, that cells with increasing mean $F_I$ levels have increasing mean MM. This now can be tested directly using a fluorescence microscope with a camera and cell imaging software that discriminates between the cell $F_I$ levels.

Third, the eqn (4.4) links three physical parameters of the labeled cells, its magnetophoretic mobility (MM), the fluorescence intensity ($F_I$) and the square of its radius ($R$). All three parameters are measured by the Fl/VIS CTV, the cell hydrodynamic radius being measured indirectly by measuring the cell sedimentation velocity. This allowed us to keep the internal consistency of the method by calibrating the cell MM against its sedimentation velocity. In particular, the error propagation formula applied to eqn (4.4) shows that the relative error of cell MM is directly proportional the relative error of cell $R$:

$$\frac{\sigma_m}{\langle m \rangle} = \sqrt{2} \frac{\sigma_R}{\langle R \rangle}$$

where $\langle m \rangle$ and $\sigma_m$ are the mean and standard deviation of the cell MM, and $\langle R \rangle$ and $\sigma_R$ are the mean and standard deviation of the cell radius. As the relative error of cell $R$ determined by the sedimentation velocity measurement is expected to be independent of changes in the labeling conditions, any experimentally measured changes in its value indicate experimental artifacts. The eqn (4.5) indicates that the experimental error contribution to the MM measurement is directly proportional to the error of the $R$
measurement (from the cell sedimentation velocity measurement). The latter is known from the changes compared to a baseline value (such as from the unlabeled cell sedimentation velocity measurement).

The cell sedimentation velocity, \( u_g \), or sedimentation coefficient, \( s \), has been introduced earlier,

\[
s = \frac{u_g}{g} = \frac{(\rho_c - \rho_f) V_c}{6\pi R \eta} = \frac{2\Delta \rho R^2}{9\eta}
\]  

(4.6)

where \( g \) is the standard gravitational acceleration, \( \rho_c \) and \( \rho_f \) are the densities of cell and suspending fluid.

**4.2.2 Determination of \( \theta_1 \) and \( \theta_2 \) in eqn (4.4)**

First, for a given cell type and antibody combination, we will determine the saturating amounts of the primary antibody using flow cytometry, as described earlier (Leigh et al. 2005). If the FI for a specific primary antibody concentration is divided by the highest FI measured in that set of experiments, then the normalized value is equivalent to \( \theta_1 \) in eqn (4.1), which is the fraction of the targeting antigen molecules that are bound by the primary antibody.

\[
\theta_1 = \frac{FI}{FI_{\text{max}}}
\]  

(4.7)

The selection of the cell type and the antibody combination fixes the parameters \( n_1, \lambda_1, n_2, \lambda_2, n_3 \) and \( I_0 \) in eqn (4.4). For other parameters being constant, at the specific concentrations of both antibodies, one obtains

\[
\theta_1 \cdot \theta_2 = \frac{m}{m_{\text{max}}}
\]  

(4.8)
Therefore, one could easily evaluate $\theta_2$, the fraction of the $n_2$ fluorescent molecules binding the tagging (secondary) antibody.

### 4.3 Materials and methods

#### 4.3.1 Visible and fluorescent version of CTV (VIS CTV and Fl CTV)

The microscope employed internal illumination (epi-illumination) by a 100-W mercury burner, and used a 5× microscope objective and 2.5× photo eyepiece (model U-MNIB, Olympus, Tokyo, Japan). Three different filter cubes allowed viewing by brightfield, darkfield and fluorescent field illumination, Figure 4.4. The CTV system discussed previously (Chapter 3) is a visible version (VIS CTV), as a darkfield microscopy rendering the samples clearly visible (dark background and bright objects). When the fluorescent filter cube is applied to the CTV, it becomes a fluorescent version (Fl CTV) to detect PE fluorescence signals. The fluorescent imaging cube contained a blue excitation filter (470 - 490 nm), a barrier filter transmitting from 510 nm upward, and a dichroic mirror transmitting from 505 nm upward. The other parts (including spatial distribution of magnetic field and gradient) of the CTV instrument were kept the same. A rectangular borosilicate glass channel (VitroCom, Mountain Lakes, NJ) was placed in a specially designed permanent magnet assembly, which produces a magnetic field induction, $B_0$ of 1.41 T and a constant magnetic energy gradient, $S_m$ of 140 TA/mm² in the viewing area (1.72 mm × 1.27 mm, width × height). The cell suspension was pumped to this glass channel and individual cell movements were observed by the microscope and a high-sensitivity, monochrome CCD camera (Retiga EXi, QImaging, Surrey, BC, Canada). Real-time disk recording software was used to control the camera
and acquire the images to PC (Video Savant 4, IO Industries, London, Ontario, Canada). The nearly constant horizontal velocity induced by the magnetic field and the vertical velocity induced by gravity of every recorded individual cell in the viewing area were calculated by the CTV software (Appendix A).

A cell sample is first detected by using the darkfield illumination in the VIS CTV system. Then, through the use of excitation and emission filters, the same cell sample is detected again by Fl CTV. Two sets of MM data of the same cell sample are compared.

Figure 4.4 VIS and Fl CTV system
4.3.2 Jurkat cell line

Jurkat, a human T cell lymphoblast-like cell line was used as the cell model due to its high and uniform surface expression of CD45 marker under normal culture conditions. The cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS) (Serum Source International, NC, USA) and 2 mM L-glutamine at 37°C, 5% CO₂.

4.3.3 Cell labeling reagents

One primary antibody from Invitrogen (Carlsbad, CA) was human CD45-R-PE (Cat. No. MHCD4504-4). Three types of magnetic colloids were used as the secondary antibody: MACS® Anti-PE Microbeads (Cat. No. 130-048-801, Miltenyi Biotec, CA, USA); BD Imag™ Anti-R-PE particles-DM (Cat. No. 557899, BD Pharmingen, CA, USA); and EasySep® PE Selection Kit (PE Selection Cocktail + magnetic nanoparticles) (Cat. No. 14852, StemCell Technologies, Vancouver, Canada).

4.3.4 Cell samples preparation for CTV analysis

A two-step labeling protocol was used to prepare four cell subpopulations for CTV analysis. As a targeting antibody, an anti-CD45 monoclonal antibody conjugated to a fluorochrome (phycoerythrin, PE), was used to illuminate the target cell population. For magnetic tagging, a secondary anti PE antibody conjugated to magnetic nanoparticles was used. Cell suspension, as collected after 2 - 3 days in culture, was centrifuged and the primary antibody, anti CD45-PE, at a specific concentration (e.g. 10 µL/10⁶ cells) was added to the cell pellet, followed by 30 min incubation on ice. Cells were washed once with the labeling buffer containing 1× PBS, 2 mM EDTA (Ethylene Diamine Tetraacetic Acid), and 0.5% BSA (Bovine Serum Albumin), and the secondary antibody, anti-PE
magnetic colloid with the same concentration of the primary antibody (e.g. 10 µL/10⁶ cells) was pipetted to the cell pellet. The cell mixture was subsequently incubated for 15 minutes on ice, followed by one washing step. For CTV analysis, the cells were diluted to about 0.5 × 10⁶ cells/mL. At this concentration, no detectable cell-cell interaction was found (data are not shown here). Meanwhile, another aliquot of cell suspension was collected and centrifuged. Then ant-PE magnetic colloid at a specific concentration (e.g. 10 µL/10⁶ cells) was added directly to the cell pellet, followed by 15 min incubation on ice to evaluate nonspecific labeling: that is, the specificity of the anti-PE magnetic colloid.

Negative control for CTV analysis was prepared, too. Cells without any labeling treatment were washed and resuspended in the cell labeling buffer (containing 1× PBS, 2 mM EDTA, and 0.5% BSA) with a concentration of about 0.5 × 10⁶ cells/mL.

4.3.5 Primary antibody saturation studies

For primary antibody labeling studies, aliquots of cell suspensions with 2 × 10⁶ cells were pelleted by centrifugation. An appropriate amount of the primary antibody, anti CD45-PE, was added to each aliquot and incubated for 30 min on ice. Afterward, cells were washed once by centrifugation and then fixed using 1% v/v formaldehyde (Cat. No. 18814, Polysciences, Inc., Warrington, PA, USA) in PBS solution. Samples were covered with aluminum foil to prevent fluorochrome bleaching and stored at 4°C until FCM analysis.

4.3.6 Identify the maximum MM of a magnetic tagging antibody achieved

The CTV software identifies the cells from the background based on their binarized grayscale intensities (0-255, black-white). Cells with gray values equal to or
higher than a threshold are identified, and their corresponding MMs and settling velocities are then calculated by the CTV software. Cells stained with more primary antibodies conjugated with fluorochrome are brighter and have higher gray value in the Fl CTV system. Meanwhile, such cells will have higher numbers of secondary antibody conjugated to magnetic beads, which induces higher MM for these cells. The grayscale threshold could not be increased without limit, because fewer cells can be detected as it is raised. Usually the threshold should be set low enough to track all of the cells in the focal plane, but high enough to exclude the background.

At a given secondary antibody concentration, a series of MM values were obtained at a series of threshold values from low to high. In a plot of the MM against the threshold value, maximum MM is found at the point where the MM curves at different concentrations merge together.

4.4 Results and discussion

4.4.1 Determination of MM₁ and MM₂ of for magnetic tagging reagents

The MMs of a negative control (unlabeled Jurkat cells) were distributed normally as shown in Figure 4.5. The magnetic cut-off mobility (MM₁) of $1.0 \times 10^{-5} \text{ mm}^3/\text{T-A-s}$ was selected at the 95th percentile of cumulative mobility frequency distribution of the negative control cells. A dot plot of MM against sedimentation coefficient of two Jurkat cell populations is shown in Figure 4.6 (A). The black dots represent the unmanipulated, negative control cells (without primary and secondary antibodies); the red dots represent Jurkat cells after incubation with the MACS® Anti-PE Microbeads only. As can be observed, red dots have a much broader mobility range. But most of the black and red
dots overlap in the region of zero magnetophoretic mobility, as expected. However, a small fraction of red cells appear above the overlap region, or above the cutoff line, $\text{MM}_1$, representing the cell fraction that binds the immunomagnetic reagent non-specifically. This is further illustrated in Figure 4.6 (B) (MM histograms). The mean MM of cells above $\text{MM}_1$ is $2.10 \times 10^{-5}$ mm$^3$/T-A-s, indicated by a second line in Figure 4.7, which is referred to as “the mean nonspecific mobility”, $\text{MM}_2$. The specific values of $\text{MM}_2$ for three magnetic colloids used in this study at different concentrations are listed in Table 4.1.

![Figure 4.5 A histogram of MM distribution of the negative control](image-url)

Table 4.1

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Frequency</th>
<th>Cumulative Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0e-5 mm$^3$/TAs</td>
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<td>0.00</td>
</tr>
<tr>
<td>2.0e-5 mm$^3$/TAs</td>
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<td>0.02</td>
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<tr>
<td>0.0 mm$^3$/TAs</td>
<td>0.04</td>
<td>0.06</td>
</tr>
<tr>
<td>2.0e-5 mm$^3$/TAs</td>
<td>0.06</td>
<td>0.12</td>
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<tr>
<td>4.0e-5 mm$^3$/TAs</td>
<td>0.08</td>
<td>0.20</td>
</tr>
<tr>
<td>6.0e-5 mm$^3$/TAs</td>
<td>0.10</td>
<td>0.30</td>
</tr>
<tr>
<td>8.0e-5 mm$^3$/TAs</td>
<td>0.12</td>
<td>0.42</td>
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</table>

Figure 4.5  A histogram of MM distribution of the negative control
Figure 4.6  (A) A dot plot of the magnetophoretic mobility against sedimentation coefficient of two Jurkat cell samples: one is negative control, and the other one was labeled with the secondary antibody only; (B) Histograms of magnetophoretic mobility distribution
4.4.2 Specificity and sensitivity of the immunomagnetic reagent.

Figure 4.7 shows MM against sedimentation coefficient for one Jurkat cell population labeled with anti CD45-PE and MACS anti-PE microbeads. Black dots represent the data from VIS CTV, and red dots are data from Fl CTV. By applying the two cutoff lines, MM1 and MM2, five cell subpopulations are quantified: A1 := {VIS MM < MM1} = (FM' + FM'M'); A2 := {Fl MM < MM1} = FM'M; A3 := {MM1 < Fl MM < MM2} = FM'M', A4 := {MM < VIS MM < MM2} = (FM' + FM'M'), and A5 := {VIS MM > MM2} = FM'. The fractional sizes of the sets, ||A1|| + ||A4|| + ||A5|| = 1, and ||A2|| + ||A3|| + ||A5|| = x, which is the fluorescently labeled cell fraction. The sensitivity of the magnetic tagging reagent was defined as ||A3|| + ||A5||. For the particular combination of reagents used in this study, ||A1|| = 1.44%, ||A2|| = 0.34%x, ||A3|| = 0.77%x, ||A4|| = 1.88%, and ||A5|| = 96.68%. Therefore, the sensitivity was equal to 97.43%. The specificity was 98.87%, as expected for a nearly homogeneous cell population. The specificity and sensitivity of the three magnetic colloids used in this paper at different concentration is shown in Table 4.1.
Figure 4.7 A dot plot of the magnetophoretic mobility against sedimentation coefficient of the two-step labeled Jurkat cells detected by VIS and Fl CTV
<table>
<thead>
<tr>
<th>Magnetic colloid</th>
<th>Ab Concentration (µL/10^6 cells)</th>
<th>Mean MM by VIS CTV (mm^3/TAs)</th>
<th>MM_1 (mm^3/TAs)</th>
<th>MM_2 (mm^3/TAs)</th>
<th>Specificity (%)</th>
<th>Sensitivity (%)</th>
</tr>
</thead>
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<tr>
<td>MACS</td>
<td>2</td>
<td>2.07e-4</td>
<td>1.00e-5</td>
<td>2.10e-5</td>
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<td>98.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.53e-4</td>
<td>1.00e-5</td>
<td>2.10e-5</td>
<td>99.6</td>
<td>98.9</td>
</tr>
<tr>
<td>BD Imag</td>
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<td>1.00e-5</td>
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<td>100</td>
<td>98.8</td>
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<td>3.00e-5</td>
<td>99.5</td>
<td>98.0</td>
</tr>
</tbody>
</table>

Table 4.1  Specificity and sensitivity of three commercial magnetic colloids after a two-step labeling procedure. (the ratio of primary and secondary Ab is 1:1)
4.4.3 θ₁ and θ₂ value

Figure 4.8 is the relative FI of the primary antibody-fluorochrome conjugate ($FI/I_0$), anti CD45-PE, as a function of the amount of antibody used per $10^6$ Jurkat cells. The FI of negative control (cells without any labeling) was normalized to 1 ($I_0 = 1$). From this saturation binding curve, a saturating concentration of $10 \mu$L/$10^6$ cells was selected.

Figure 4.9 is an example of a typical set of curves of MM at different concentration of secondary magnetic tagging antibody as a function of grayscale threshold in CTV. A maximum MM at around $6.20 \times 10^{-4}$ mm$^3$/TAs was determined. It also suggests that the concentration at about $14 \mu$L/$10^6$ cells is the saturating concentration of the secondary antibody.

![Figure 4.8](image-url)

Figure 4.8 Saturation binding curve of Jurkat cells stained with varying amounts of anti-CD45-PE antibody, measured by cell fluorescence intensity which was presented here relative to the cell fluorescence intensity of negative control (cells without any labeling)
Then from eqn (4.7), $\theta_1 \cdot \theta_2$ at varying concentrations of the secondary antibody was calculated and shown in Figure 4.10. For example, if the concentration of 9 uL/10^6 for the primary antibody (almost saturated) was selected, the value $\theta_1 \approx 1$ was obtained from eqn (4.6) and Figure 4.8. When 9 uL/10^6 cells of the secondary antibody was also used, from Figure 4.10 (gray dot line), one obtains mean $\theta_1 \cdot \theta_2 \approx 0.4$. Then mean $\theta_2$ equal to 0.4 was obtained. This indicates that specific cell antigens were all bound with the primary antibodies, but only about 40% of the primary antibodies were occupied by the secondary magnetic tags.
Figure 4.10 $\theta_1 \ast \theta_2$ curves of five Jurkat cell samples labeled with five different amounts of antibodies

### 4.5 Conclusions

A combination of VIS and Fl CTV analyses allowed us to compare MM of four different cell populations: unlabeled cells, cells that bind primary antibody but not the magnetic colloid, cells that bind both the primary antibody and the magnetic colloid, and cells that bind the magnetic colloid only. The presence of cells that bind the magnetic colloid only determines less than ideal specificity of the labeling procedure. The presence of cells that bind both the primary antibody and the magnetic colloid determines the sensitivity of the labeling procedure. The results of tests performed on homogeneous cell mixture showed high specificity and high sensitivity of all three types of labeling.
reagents, as expected. The method is currently applied to determine specificity and sensitivity of newly synthesized magnetic colloids.

Moreover, with the capacity of detecting fluorescence signal, CTV analysis could differentiate MMs based on the binary grayscale threshold. Cells stained with more fluorescent primary antibodies are brighter which corresponds to higher gray values in the tracked images (black-white color style: 0, black, to 255, white). Subsequently, brighter cells yield more bound secondary magnetic-bead conjugates, which induces higher MM for these cells. Combining with FCM, FL CTV is capacity of determining $\theta_1$ and $\theta_2$, the fraction of the targeting antigen molecules bound by the primary antibody, and the fraction of binding sites on the primary antibody occupied by the secondary antibody.
CHAPTER 5

CULTURED RED BLOOD CELL SEPARATION

5.1 Introduction

Red blood cells (RBCs), which make up 40 percent of the average human's blood volume, are the most commonly transfused blood product, with 40,000 RBC units (~220 mL) used in the United States every day (Sullivan et al. 2007). But problems such as constant difficulty in obtaining adequate supplies, lack of availability of certain phenotypes, and possibility of infectious agents transmission to the recipients create the need for alternative sources of RBCs for transfusion. A “blood pharming” program led by Defense Advanced Research Projects Agency (DARPA) contracted to Celgene Cellular Therapeutics (CCT) that has for its goal a large scale in vitro production of RBCs from hematopoietic stem cells (HSCs) isolated from umbilical cord and placental blood discarded following delivery, could be a promising new solution to provide a ready supply of safe and effective RBCs for transfusion. The HSCs are identified by a cluster of differentiation 34 (CD34) surface marker and isolated immunomagnetically from cord and placental blood, then cultivated in standard 2D culture or novel multiple bioreactors
that can mimic bone marrow microenvironment (Gerlach et al. 2010). After substantial expansion without differentiation, HSCs are induced to differentiate into mature, functional RBCs (Delaney et al. 2005; Giarratana et al. 2005). During differentiation (Figure 5.1), erythroid cells progressively become smaller and exhibit chromatin condensation, increased hemoglobin concentration, exit the cell cycle, and become enucleated. Due to such a complex process of mammalian erythropoiesis and because of multipotency of HSCs, it is very difficult to constrain a HSC to commit itself exclusively to the erythroid line and homogeneously differentiate and mature into an enucleated RBC.

![Figure 5.1 Mammalian erythropoiesis (Marieb and Hoehn 2007)](image_url)

In order to ensure efficient separation of mature RBCs from the HSC culture suspension, Drs. Chalmers and Zborowski (advisors) proposed to use magnetic separation of cultured RBCs by exploiting the paramagnetic nature of deoxygenated hemoglobin (deoxy Hb). As early as 1936 Pauling has found that deoxygenated hemoglobin and methemoglobin (metHb) are paramagnetic (Pauling and Coryell 1936a) because of the existence of unpaired electrons in the four heme groups. In contrast, due to its covalent
bonds, oxygenated hemoglobin (oxy Hb) has no unpaired electrons and is diamagnetic. High gradient magnetic separators were used to directly enrich the RBCs from the whole blood deoxygenated by nitrogen gas (Furlani 2007; Han and Frazier 2004; Han and Frazier 2006; Takayasu et al. 1982; Takayasu et al. 2000). In this study, a commercial magnetic separation system, MACS LD columns in combination with a QuadroMACSTM separator from Miltenyi Biotech, was used to isolate mature RBCs from the harvested CCT cultures. The unique specifications of LD columns, given by specific shape and matrix, result in a slower flow rate and higher retain efficiency for weakly magnetic cells as compared to other MACS columns. Using cell tracking velocimetry (CTV), an analytical instrument developed in our laboratories, I was able to measure experimentally differences in RBC magnetophoresis pre and post separation, which allowed me to determine the separation performance. I also measured other physical parameters that distinguish mature RBC from immature cells in the HSC culture, such as size, and morphology. Additional tests on magnetically enriched mature RBC fractions were performed by collaborating laboratories, including cell deformability and hemoglobin concentration.

5.2 Theory

5.2.1 Red blood cell magnetic susceptibility

According to Zborowski et al. (Zborowski et al. 2003), the RBC volume magnetic susceptibility is the weighted sum of the susceptibilities of its components

\[ \chi_{RBC} = \phi_{H_2O} \chi_{H_2O} + (1 - S) \phi_{Hb} \chi_{deoxyHb} + \phi_{Hb} \chi_{globin} \]  

(5.1)
where $\phi_{H2O}$, $\phi_{Hb}$ are the volume fractions of water and hemoglobin in the RBC, respectively, $\chi_{H2O}$ (-0.719 x $10^{-6}$, cgs, or -9.04 x $10^{-6}$, SI), and $\chi_{deoxyHb}$, and $\chi_{globin}$ are the volume magnetic susceptibilities of water, the four ferroheme groups in deoxy Hb, and globin, respectively. $S$ is the oxygen saturation of oxyhemoglobin, $S = 1$ for a fully oxygenated RBC (oxy RBC), $S = 0$ for a fully deoxygenated RBC (deoxy RBC).

The volume fraction taken up by water in a RBC is determined from the volume fraction of the hemoglobin,

$$\phi_{H2O} = 1 - \phi_{Hb} = 1 - V_{m,Hb}c_{Hb} = 0.735$$  \hspace{1cm} (5.2)

where $V_{m,Hb} = 48.227$ L/mol is the molar volume of hemoglobin, and $c_{Hb} = 5.5 \times 10^{-3}$ mol/L (corresponding to 350 g/L with a molecular weight $M_{w,Hb} = 64,450$ g/mol) is the intracellular hemoglobin concentration in one mature RBC.

The volume magnetic susceptibility of metHb RBC has a similar form as the one above but contributed mainly by metHb:

$$\chi_{RBC} = \phi_{H2O}\chi_{H2O} + Z\phi_{Hb}\chi_{metHb} + \phi_{Hb}\chi_{globin}$$  \hspace{1cm} (5.3)

where $Z$ is the fraction of Hb converted to metHb, and $\chi_{metHb}$ is the magnetic susceptibility of methemoglobin.

The numerical values of the remaining terms in eqns (5.1) and (5.3) are as follows:

$$\phi_{Hb}\chi_{deoxyHb} = c_{Hb}\chi_{m,deoxyHb}$$  where $\chi_{m,deoxyHb} = 50,893 \times 10^{-9}$ L/mol is the molar susceptibility of deoxy Hb, $\phi_{Hb}\chi_{globin} = c_{Hb}\chi_{m,globin}$, where $\chi_{m,globin} = -37,832 \times 10^{-9}$ L/mol (Cerdonio et al. 1985) is the molar susceptibility of the globin, and $\phi_{Hb}\chi_{metHb} = c_{Hb}\chi_{m,metHb}$, where $\chi_{m,metHb} = 56,000 \times 10^{-9}$ L/mol is the molar susceptibility of met Hb (Coryell et al. 1937; Hackett et al. 2009). By inserting these terms into Eqns. (5.1) and (5.3), one
obtains the volume magnetic susceptibilities for RBCs in cgs unit system, which could be converted to SI unit system by multiplication of \(4\pi\):

\[
\chi_{\text{oxy}RBC} = -7.36 \times 10^{-7} \text{ (cgs)} \text{ or } -9.25 \times 10^{-8} \text{ (SI)}, \text{ fully oxy RBC, } S = 1
\]

\[
\chi_{\text{deoxy}RBC} = -4.56 \times 10^{-7} \text{ (cgs)} \text{ or } -5.74 \times 10^{-6} \text{ (SI)}, \text{ fully deoxy RBC, } S = 0
\]

\[
\chi_{\text{metHb}RBC} = -4.28 \times 10^{-7} \text{ (cgs)} \text{ or } -5.38 \times 10^{-6} \text{ (SI)}, \text{ fully metHb RBC, } Z = 1
\]

Note that diamagnetic contributions to the RBC magnetic susceptibility predominate, even for deoxy Hb and met Hb RBCs.

### 5.2.2 Magnetophoretic mobility

Magnetophoretic mobility (MM), \(m\), is defined as a ratio of the field-induced velocity, \(u_m\), and the local magnetostatic energy density gradient, \(S_m\):

\[
m = \frac{u_m}{S_m}
\]

For paramagnetic and diamagnetic cells, it reduces to an expression that depends only on cell and the suspending fluid properties:

\[
m = \frac{(\chi_c - \chi_f)V_c}{3\pi D_c \eta}
\]

where \(\eta = 0.93 \times 10^{-3}\text{ kg/m-s}\) is aqueous solution viscosity, \(V_c\) and \(D_c\) are cell volume and hydrodynamic diameter, \(\chi_c\) and \(\chi_f\) are volume magnetic susceptibility of a cell and media, respectively. Here \(\chi_f = -9.04 \times 10^{-6}\). Notably, \(\chi_{\text{oxy}RBC} - \chi_f < 0\) for the fully oxygenated RBC, and \(\chi_{\text{deoxy}RBC} - \chi_f > 0\), \(\chi_{\text{metHb}RBC} - \chi_f > 0\) for the fully deoxygenated and methemoglobinized RBC (see above). Consequently, we expect that the oxy RBCs in
solution are pushed away by the magnet (with negative MM value), and the deoxy RBCs and metHb RBCs are attracted by the magnet (with positive MM value).

### 5.2.3 Sedimentation coefficient

Considering a simple spherical cell falling through a motionless fluid, its immersed weight can be balanced by the drag force which is given by the Stokes equation.

\[
\left( \rho_c - \rho_f \right) g V_c = 3 \pi D_c \eta u_g
\]  

(5.6)

The particle sedimentation coefficient, \( s \), is then defined as

\[
s = \frac{u_g}{g} = \frac{\left( \rho_c - \rho_f \right) V_c}{3 \pi D_c \eta}
\]  

(5.7)

where \( u_g \) is the settling velocity, \( g = 9.81 \text{ m/s}^2 \) is the standard gravitational acceleration, \( \rho_c \) and \( \rho_f \) are densities of the cell and suspending medium.

The diameter of a sedimenting cell can be calculated by measuring cell sedimentation velocity, or its sedimentation coefficient,

\[
D_c = \left[ \frac{18 \eta u_g}{g \left( \rho_c - \rho_f \right)} \right]^{1/2}
\]  

(5.8)

CTV is capable of measuring \( m \) and \( s \) simultaneously for hundreds to thousands cells on a cell by cell basis, which allows us to differentiate small, magnetic RBCs or RBC-like cells from bigger, diamagnetic non-RBC cells or undifferentiated, erythroid cells (see Figure 5.1).
5.2.4 Estimation of intracellular Hb concentration in cultured RBCs

The parameter \( m \) of a deoxy or metHb RBC is directly proportional to cell magnetic susceptibility (eqn (5.5)), which is a linear function of the cell deoxy Hb or metHb content (eqn (5.1) or (5.3)). When a deoxy Hb or metHb containing RBC is placed in the CTV system, where the magnetic force is orthogonal to gravity, it moves in the horizontal direction caused by magnetic energy density gradient, eqn (5.5), as well as vertical direction induced by gravitational acceleration, eqn (5.7). Dividing eqn (5.5) by eqn (5.7), one eliminates \( \eta, D_c, \) and \( V_c \) to obtain:

\[
\chi_{RBC} = \frac{m(\rho_s - \rho_f)}{s} + \chi_f
\]  

(5.9)

Then combining eqn (5.1) or (5.3) with eqn (5.9), one obtains expression for Hb concentration for a single cultured RBC if density of RBC and physical properties of medium are known.

\[
c_{Hb} = \frac{\chi_{RBC} - \chi_{H_2O}}{\chi_{m, metHb} + \chi_{m, globin} - V_{m, Hb} \chi_{H_2O}} \quad (mol / L)
\]

(5.10)

With the molecular weight of hemoglobin (\( M_{w, Hb} = 64,450 \) g/mol) and the measured cell volume data, one can obtain a value for mean corpuscular hemoglobin (MCH, pg/RBC), which is the average mass of hemoglobin per RBC, or mean corpuscular hemoglobin concentration (MCHC, g/dL RBC), which is the concentration of hemoglobin in a given volume of packed RBC. In this manner, the RBC magnetophoresis measurement by CTV is capable of providing information on hemoglobin concentration in mature, cultured RBC that is accumulated gradually in during erythropoiesis.
5.3 Materials and methods

5.3.1 Cell sources

Cell cultures in the original culture media were obtained from Celgene Cellular Therapeutics (CCT). Once received, CCT cultures were centrifuged once at 250g for 10 minutes. The cell pellet was then suspended in Ca\(^{2+}\), Mg\(^{2+}\)-free Dulbecco’s phosphate-buffered saline (PBS) containing 0.5% Bovine Serum Albumin (BSA, Cat. No. A-3059, Sigma, St. Louis, MO) and 2 mM Ethylene Diamine Tetraacetic Acid (EDTA, Cat. No. E9884, Sigma, St. Louis, MO). The cell suspension was filtered by a 40 µm cell strain (Cat. No. 352340, BD Biosciences, Durham, NC) to remove cell aggregates. The cell concentration was determined and a desired concentration for separation was prepared.

The Whole Blood (WB) was received from the Cleveland Clinic Blood Banking and Transfusion Medicine under an Institutional Review Board (IRB) approved protocol for blood collection from normal volunteers for research. A stock suspension was prepared by diluting 0.1 mL whole blood with 10 mL PBS. An aliquot of \(2.0 \times 10^6\) RBCs from the stock suspension was used as the donor-derived oxy RBC control (negative control).

5.3.2 Irreversible RBC magnetization

A 5 mM oxidant solution was prepared by dissolving sodium nitrite (NaNO\(_2\), Cat. No. 524379, Sigma-Aldrich Co., Milwaukee, WI) in PBS at room temperature. An aliquot of \(2.5 \times 10^6\) RBCs from the RBC stock suspension prepared as described above was centrifuged and resuspended in 10 mL of 5 mM sodium nitrite solution, which was then incubated for about 1.5 hours to achieve a 100% methemoglobin oxidation. An
aliquot of CCT cultures with same cell number was oxidatively treated at the same way: suspended in 10 mL of 5 mM NaNO₂ solution, 1.5 hours. After incubation, methemoglobinated RBCs or CCT cultures were washed once and resuspended in 5 mL PBS containing 0.1% Pluronic F-68 (Cat. No. P-1300, Sigma-Aldrich, St Louis, MO) for CTV analysis. Methemoglobinated donor-derived RBC was regarded as positive control.

5.3.3 Deoxygenation of CCT cultures - reversible RBC magnetization

A Glove-Bag™ inflatable glove chamber (Cat. No. K-04408-34, Cole Parmer, Vernon Hills, IL), filled with nitrogen (Medipure™ nitrogen, concentration > 99%, Praxair, Inc., Danbury, CT) was used to deoxygenate CCT cultures, Figure 5.2. It was sterilized by 70% alcohol and kept in a laminar flow biosafety hood. Before deoxygenation, all materials and equipments including the separation system, degassed sterile buffer (PBS + 2 mM EDTA + 0.5% BSA), and sterile collection tubes were placed in the glove bag, which was then tightly sealed.

The nitrogen gas was humidified by bubbling through water. After passing a 0.2 µm filter, the humidified gas entered into the glove bag through a tubing coupler, which was inserted into the gas port built into the glove bag. The actual gas flow rate into the glove bag was not measured, but the gas pressure was controlled by a N₂ gas cylinder pressure regulator at about 50 kPa.
The deoxygenation system efficiency in removing O$_2$ from blood was first tested on 5 mL WB placed in an inclined 50 mL conical tube affixed to a stirring rotator in N$_2$ atmosphere. The relatively large surface area of the 50 mL conical tube and its rotating motion facilitated a rapid gas exchange in a small sample volume smeared over the internal surface of the conical tube. About 0.1 mL sample were taken at different time points for blood oximetry. Partial pressure of oxygen (pO$_2$) and oxygen saturation (sO$_2$) were measured by a blood gas analyzer (ABL715, Radiometer, Denmark). A practical
exposure N₂ time was selected and then applied to cultured RBC samples based on these results.

5.3.4 Magnetic cell separation

A commercial magnetic separation system (QuadroMACSTM Separator combining four MidiMACSTM separation units and LD columns, Miltenyi Biotec, Auburn, CA) was used for magnetic RBC enrichment from CCT cultures. Deoxygenated CCT cultures were loaded directly into a MACS® LD column which was placed in the QuadroMACSTM separator. Cells passed through the column while on the magnet are labeled as negative fraction and they are expected to be “non-magnetic” including HSCs and erythroid cells before final maturation. The cells retained in the separation column are labeled as positive fraction, which is “magnetic” and expected to consist of mature RBC-like cells full of functional hemoglobin. They were eluted from LD column after its removal from the magnet. In order to increase mature RBC recovery, another column was used to repeat the separation process (dubbed “two-stage”) using negative fraction as a feed. Positive fractions from these two separations were pooled. One-stage and two-stage separation processes were shown schematically in Figure 5.3. Once separation was finished, oxygenated cells were reversibly recovered by exposing the collected cells to air.
5.3.5 CTV analysis

The CTV instrument was used, as described in Chapter 1, to measure magnetophoretic mobility and settling velocity of the donor-derived RBC controls, oxy RBC and metHb RBC. The cell number concentration was kept at $0.4 \times 10^6$ cells/mL. The unsorted and sorted RBC fractions were exposed to oxidative treatment to convert hemoglobin to methemoglobin in order to be able to identify the hemoglobin-containing cells (the putative mature RBCs) by CTV analysis, and to evaluate the separation performance. More than 1,000 cells in each sample were recorded and analyzed.

5.3.6 Cell concentration and size distribution

An automated cell counter, Z2™ Coulter Counter® (Beckman Coulter Inc., Fullerton, CA, USA), with a 70 µm aperture, a sample volume of 0.5 mL and a diameter
range setting between 4 and 14 µm was used to measure cell concentration and size distribution.

5.3.7 Cell morphology analysis by differential interference contrast (DIC) microscopy

Ten µL of sample was placed on a slide and covered by a cover slip, which was then visualized by a Leica DMR upright microscope equipped with a 40× objective lens and a CCD camera provided by the Imaging Core of Lerner Research Institute in the Cleveland Clinic. The software, ImagePro Plus was used to acquire and process the DIC images. Unsorted and sorted samples were analyzed and compared. The RBC images from donor blood were used as a control.

5.3.8 RBC deformability

Mature RBCs have the ability to deform under the influence of externally applied shear stresses. RBC deformability was determined under fluid shear stress by ektacytometry. This analysis was performed in Dr. Marina Kameneva’s lab, University of Pittsburgh (Lee et al. 2007).

5.4 Results and discussion

5.4.1 Deoxygenation of whole blood

Deoxygenation of 5 mL whole blood in one 50 mL rotating conical tube was tested in the N₂ filled glove bag. After 3 hr exposure to N₂, blood oxygen partial pressure (pO₂) dropped to 2.3 mmHg (the pO₂ in room air is approximately 156 mmHg), blood oxygen saturation (sO₂) dropped to 3.9% (from the initial value of 99%), as shown in
Figure 5.4. The mean RBC number concentration is $5 \times 10^9$/mL of whole blood, which is much higher than that expected in CCT cultures and therefore the same or better deoxygenation results were expected for the CCT cultures. Consequently, 5 mL CCT cultures with a cell number concentration of $(1-2) \times 10^8$/mL were placed in the same type of 50 mL, inclined, rotating conical tube and exposed to N$_2$ atmosphere for 3 hr, following which all mature RBCs in CCT culture were considered to be deoxygenated completely. After deoxygenation, CCT cultures were isolated by MACS separation in the glove bag, as described above.
5.4.2 Differences in MM of oxygenated and metHb RBCs from donor blood

As stated earlier in the introduction section, the magnetic susceptibility of erythrocyte is related to the hemoglobin magnetic susceptibility which, in turn, depends on oxygen binding to the heme group. Oxygenated RBC is more diamagnetic than the aqueous physiologic electrolyte solution while metHb-containing RBC is less diamagnetic than the aqueous media due to paramagnetic contribution of the metHb, resulting in negative MM of oxy RBC and positive MM of the metHb RBC, as discussed earlier. The theoretical predictions described in Sections 5.2.1 and 5.2.2 were borne out by my experimental results. Figure 5.5 shows the histograms generated by CTV measurements of the MM of these two RBCs. A noticeable shift toward higher MM values for metHb containing RBC is observed relative to the RBC equilibrated with the air. The likely source of the mobility dispersion around the mean value is the heterogeneous nature of the normal RBC population and the background noise introduced by CTV. The RBC data were used as a reference for the results of this study, specifically, the air-equilibrated (the “oxygenated”) RBC data as a negative control, and the metHb RBC data as a positive control. It is also worth noting the high sensitivity of the CTV instrument, sufficient to detect changes in the individual cell motion caused by changes in the intracellular iron spin state. Such high sensitivity is necessary for even more challenging projects on magnetophoretic mobility analysis of cancer cell lines, pursued in Chapter 7.
5.4.3 CTV as a tool for determination of RBC-like cells in HSC cultures

Before separation, an aliquot of CCT cultures was oxidatively treated to convert the hemoglobin to methemoglobin in the hemoglobin-containing cells. These cell preparations were then analyzed by CTV for MM and sedimentation rate. Figure 5.6 shows the MM histograms of the cell culture before and after oxidative treatment. Note
shift in the mobility distribution to the right, as expected of a sample preparation with
cells that contain metHb. This indicated presence of cells with hemoglobin (mature
RBC’s and reticulocytes, Figure 5.1) in the CCT cultures. Compared with the RBC
negative (oxy RBC) and positive (metHb RBC) controls, Figure 5.5, MM of oxidatively
treated sample suggests presence of cells with a whole spectrum of the hemoglobin
concentration, from none to low to a maximum concentration characteristic of mature
RBCs.

In an attempt to improve discrimination between the Hb containing cells and other
cells, I have used both the MM (indicated by the letter $m$) and the cell sedimentation rate
to generate 2D dot plots. Cells without hemoglobin are diamagnetic. Only contribution of
paramagnetic deoxyHb or metHb makes the RBC magnetic, which was reflected in an increase in \( m \) as measured by CTV, Figure 5.6. The value of \( m \) of a deoxy or metHb containing RBC (or RBC-like cell) is directly proportional to the cell magnetic susceptibility, which is a linear function of the cell hemoglobin content, eqns (5.3) and (5.5). At the same time, the cell sedimentation velocity, also measured by CTV, is a function of the cell size, eqn (5.7). Based on the fact that the cell size and hemoglobin content in RBC and stem cell or other immature erythroid cells are different, Figure 5.1, I proposed that we can differentiate RBCs or RBC-like cells (small, paramagnetic) from the coexisting stem cells or other immature erythroid cells (large, diamagnetic) by applying quadrant statistics, in a manner that is routinely used for interpreting flow cytometry data (Shapiro and Leif 2003). This is illustrated by the dot plot for the negative and positive RBC controls, Figure 5.7. In the dot plot of \( m \) against settling velocity for RBC controls, quadrant gates are also shown in Figure 5.7, \( 2.2 \times 10^{-6} \text{ mm}^3/\text{TAs} \) and \( 2.2 \times 10^{-3} \text{ mm/s} \), respectively. Cells in the 2\text{nd} quadrant (upper left) are metHb containing RBCs. Nonmagnetic cells with similar size of RBCs locate at the 3\text{rd} quadrant (low left). Cells in the 4\text{th} quadrant (low right) are bigger than RBC and nonmagnetic. Cells containing similar amount of hemoglobin as RBC but bigger than RBC are in the 1\text{st} quadrant (upper right). Note excellent separation between negative and positive RBC control populations by the 2D dot plot analysis of the CTV data, confirming conclusions drawn from Figure 5.6.
In Figure 5.8, the same quadrant gates selected for the negative and positive RBC controls were applied to the unsorted CCT cultures which were oxidatively treated. Quadrant statistics was used to determine cell frequency distribution between different quadrants and the results are shown in Table 5.1. By this statistic, 31% of cells meeting the criteria of RBC-like cells, were gated in the 2nd quadrant, while 26% of cells meeting the criteria of non-magnetic, bigger cells were located at the 4th quadrant. There were 22% of bigger cells with similar content of hemoglobin as the mature RBCs in the 1st quadrant, and there were also 21% non-magnetic cells with similar size of RBCs in the 3rd quadrant.

Subsequently, after each separation, the positive and negative cells collected from the magnetic column were oxidatively treated and analyzed by CTV. Representative examples of dot plot of sorted fractions are shown in Figure 5.9 (A) and (B). Most cells
in the positive fraction are magnetic, which suggests that the hemoglobin containing cells were enriched from 53% to 84% (1\textsuperscript{st} + 2\textsuperscript{nd} quadrant). The RBC-like cells appearing in the 2\textsuperscript{nd} quadrant were enriched from 31% to 51%.

Figure 5.8 Dot plots of mobility vs. settling velocity for CCT culture after oxidative treatment

<table>
<thead>
<tr>
<th>quadrant</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before separation</td>
<td>22.2%</td>
<td>31.2%</td>
<td>20.8%</td>
<td>25.9%</td>
</tr>
<tr>
<td>Positive fraction</td>
<td>32.4%</td>
<td>51.4%</td>
<td>11.1%</td>
<td>5.1%</td>
</tr>
<tr>
<td>Negative fraction</td>
<td>16.3%</td>
<td>30.3%</td>
<td>28.8%</td>
<td>24.7%</td>
</tr>
</tbody>
</table>

Table 5.1 Quadrant statistics on unsorted and sorted samples shown in Figure 5.9.
On closer examination, the dot plot of positive fraction has a similar shape as that of RBC positive control, but the whole population shifts to higher settling velocity values.
This indicates that the isolated positive cells contain similar amount of hemoglobin as donor-derived RBC, but have bigger size. This observation was further confirmed by the Coulter counter and morphological analysis, described below. Cells in the negative fraction are on average less magnetic and are larger as compared to the positive fraction, Figure 5.9 (B).

5.4.4 Cell size analysis by Coulter counter and CTV

Cell size in the unsorted and sorted samples were evaluated by Coulter counter and CTV. Figure 5.10 presents the cell volume distribution of the CCT culture before and after MACS separation. The narrower distribution of the positive fraction suggests a more homogenous cell composition, consistent with the MM results, discussed above. Also notable is the mean cellular volume (MCV, ≈ 200 fL) of the positive fraction that is much bigger than that of normal donor-derived RBCs, reported in the literature, which is in the range of 80-100 fL (Lichtman et al. 2005). This agrees with the shift to the right of the positive cell fraction after magnetic separation of the CCT culture, relative to the positive RBC control, discussed above in reference to Figure 5.9. The phenomenon is further illustrated by settling velocity distributions measured by CTV shown in Figure 5.11, as the settling velocity proportional to cell size. Unsorted cells and negative cells have a broader settling velocity distribution. The selected positive cells sedimented a little faster in the gravity field than the normal donor-derived RBCs. This again indicates that the enriched cells are bigger.
Figure 5.10  Cell volume of unsorted and sorted cell samples by Coulter counter method
Figure 5.11  Settling velocity of unsorted (A) and sorted cell samples (B, positive fraction; C, negative fraction) by CTV
5.4.5 Hemoglobin content in enriched RBC-like cells

As presented in Figure 5.9 (A), small portion of diamagnetic cells were present in the enriched (positive) fraction following magnetic separation. These are most likely the non-Hb containing cells with negative MM values as they are more diamagnetic than the suspending medium (water) due to the diamagnetic properties of major components, including water, proteins, nucleic acids, carbohydrates, and lipids, as discussed earlier. It is meaningless to calculate Hb content in these non-Hb containing cells. Therefore, only gated cells (with MM > $2.2 \times 10^{-6}$ mm$^3$/TAs) in 1$^{\text{st}}$ and 2$^{\text{nd}}$ quadrants, shown in Figure 5.9 (A), were selected for Hb content calculation, using eqn (5.10) as they meet the RBC criteria, as discussed above. The mass density of these Hb containing cells was determined by applying the mean cell diameter (7.26 µm) from the Coulter counter analysis and the mean settling velocity (2.12 $\times$ $10^{-3}$ mm/s) from CTV analysis to eqn (5.6). A value of 1.070 g/cm$^3$, somewhat smaller than that of mature RBCs, was obtained, which was then used to calculate the diameter and volume of Hb containing cells from the settling velocity data. The histogram of mean corpuscular hemoglobin (MCH) with a mean value of 26.6 pg/cell and the standard deviation of 9.0 pg/cell is shown in Figure 5.12. These values are very close to the MCH values of normal donor-derived RBC, which are in a range of 27.5 - 33.2 pg/cell (Lichtman et al. 2005).
5.4.6 Morphology and deformability analysis

DIC microscopy is an optical microscopy illumination technique used to enhance the contrast in unstained, transparent cell samples in their natural milieu (aqueous solutions). The image has a very realistic, clear, three-dimensional appearance almost entirely free of the annoying phase contrast artifacts of phase halo and shading off compared to a phase contrast image (Salmon and Canman 2001). Figure 5.13 present examples of DIC images of unsorted and sorted cell samples. One notes that the original cell population of CCT culture (panel B) is heterogeneous, with a very small fraction of RBC-like cells, as compared to the blood donor RBC’s (panel A). In contrast, the morphology of the magnetically separated, positive cell fraction (panel C) is very homogeneous, with increased frequency of the small, round, RBC like cells as compared
to the original sample and the magnetic negative fraction (panel D). These results confirm the results of CTV and Coulter counter analysis, presented above.

Figure 5.13  The morphology of donor-derived RBC (A) and the CCT culture before (B) and after separation (C, positive fraction; D, negative fraction)

Selected cell aliquots were shipped overnight for deformability analysis by ektacytometry to the laboratory of Dr. Kameneva, University of Pittsburgh. Typical images produced by ektacytometry are presented in Figure 5.14. The light absorption
coefficient is relatively high for the red pigment carrying protein, hemoglobin inside a RBC as compared to water, and relatively low for other non-Hb containing cells and the suspending medium. The use of a small bandwidth interference filter (380 - 420 nm) made the Hb containing cells appear as dark objects against the bright background and other non-Hb containing cells. Again, one notes a clear evidence of enrichment of Hb containing cells that are characterized by high deformability rate were in the positive fraction following magnetic separation. These results agree with the results of the CTV analysis, discussed above, and are further proof of the accuracy of the CTV method.

Figure 5.14 Deformability of cells in positive fraction (A) and negative fraction (B). Note increased frequency of dark, elongated objects, associated with deformable, hemoglobin containing cells characteristic of mature RBC, in panel A.
5.4.7 Quantitative MACS performance characterization following separation of CCT cultures

The cell recovery in each fraction from a two-stage separation process is shown in Table 5.2. By two-stage, batch-wise magnetic column separation, the positive cell recovery was better than $3.00 \times 10^7$ out of the total of $5.00 \times 10^8$ cells applied to the column, which is the maximum capacity of one LD column recommended by the manufacturer. Overload of cells ($6.00 \times 10^8$) in experiment #4 probably explains the results of lower positive cell recovery.

<table>
<thead>
<tr>
<th>Experiment #</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed Cell # (2.5mL)</td>
<td>$5.00 \times 10^8$</td>
<td>$5.00 \times 10^8$</td>
<td>$5.00 \times 10^8$</td>
<td>$6.00 \times 10^8$</td>
</tr>
<tr>
<td>Positive fraction Cell #</td>
<td>$3.53 \times 10^7$</td>
<td>$3.49 \times 10^7$</td>
<td>$3.68 \times 10^7$</td>
<td>$2.47 \times 10^7$</td>
</tr>
<tr>
<td>Negative fraction Cell #</td>
<td>$3.58 \times 10^8$</td>
<td>$3.75 \times 10^8$</td>
<td>$3.41 \times 10^8$</td>
<td>$4.76 \times 10^8$</td>
</tr>
<tr>
<td>Overall recovery (%)</td>
<td>78.7</td>
<td>82.1</td>
<td>75.5</td>
<td>83.5</td>
</tr>
</tbody>
</table>

Table 5.2 Two-stage separation results of one CCT culture

I have determined that the process of the magnetic separation was significantly hampered by column clogging, which was then traced back to a large number of platelet-like cells found in batches of CCT cultures, which greatly reduced the column capacity. These platelet-like cells, likely immature platelets or released cell nuclei, tend to adhere to each other and are retained in the column, and subsequently are collected in the positive fraction (Figure 5.15). Severe column clogging occurred in a number of HSC
batches received from CCT if the same separation parameter were applied to all the batches \((5.00 \times 10^8\) cells per column, two-stage separation). In order to maintain similar separation throughput (cell number separated per day), I was forced to switch to the one-stage separation process, with the resulting decreased number of cells loaded per column and the feed sample volume. Even then, the LD columns clogged occasionally even for low number of loaded cells of \(2.00 \times 10^8\) total. A better overall recovery (over 90\%, as shown in Table 5.3) was obtained as compared to two-stage separation, although the throughput per column decreased significantly.

![Evidence of platelet-like cells in the positive fraction](image)

Figure 5.15 Evidence of platelet-like cells in the positive fraction
<table>
<thead>
<tr>
<th>Feed cell volume (mL)</th>
<th>Rinse #</th>
<th>Positive cell # Mean ± SD</th>
<th>Positive cell # feed cell # (%)</th>
<th>Overall recovery Mean ± SD (%)</th>
<th>CCT cultures #</th>
<th>Expt. #</th>
<th>Sorting speed (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00×10⁸/1.0 mL</td>
<td>2</td>
<td>(1.57 ± 0.12)×10⁷</td>
<td>15.7 ± 1.2</td>
<td>93.9 ± 4.8</td>
<td>3</td>
<td>14</td>
<td>1.1×10⁵</td>
</tr>
<tr>
<td>1.50×10⁸/1.5 mL</td>
<td>3</td>
<td>(1.86 ± 0.25)×10⁷</td>
<td>12.4 ± 1.7</td>
<td>98.6 ± 4.7</td>
<td>3</td>
<td>3</td>
<td>1.2×10⁵</td>
</tr>
<tr>
<td>2.00×10⁸/2.0 mL</td>
<td>4</td>
<td>(2.13 ± 0.46)×10⁷</td>
<td>10.7 ± 2.3</td>
<td>98.3 ± 3.8</td>
<td>4</td>
<td>16(3*)</td>
<td>1.2×10⁵</td>
</tr>
<tr>
<td>2.50×10⁸/2.5 mL</td>
<td>4</td>
<td>(3.44 ± 0.31)×10⁷</td>
<td>13.8 ± 1.2</td>
<td>96.4 ± 3.2</td>
<td>1</td>
<td>6(1*)</td>
<td>1.4×10⁵</td>
</tr>
<tr>
<td>2.50×10⁸/2.5 mL</td>
<td>5</td>
<td>(1.94 ± 0.13)×10⁷</td>
<td>7.8 ± 0.5</td>
<td>101.5 ± 6.5</td>
<td>2</td>
<td>7</td>
<td>1.2×10⁵</td>
</tr>
<tr>
<td>3.00×10⁸/3.0 mL</td>
<td>5</td>
<td>(2.37 ± 0.26)×10⁷</td>
<td>7.9 ± 0.9</td>
<td>98.8 ± 3.5</td>
<td>2</td>
<td>6(2*)</td>
<td>1.4×10⁵</td>
</tr>
</tbody>
</table>

(* failed experiment #, in which the column was clogged)

Table 5.3 One-stage separation results
The typical flow rate of cell suspension through the column was about 0.2 - 0.25 mL/min. The total cell sorting speed per column could be estimated and is listed in Table 5.3. The best cell recovery in the positive fraction was obtained for the feed of $1.00 \times 10^8$ cells, followed by 2 mL buffer rinse. The associated cell sorting speed per column is the worst, however. A relatively high sorting speed and a reproducible cell recovery in the positive fraction were obtained for separation conditions involving $2.50 \times 10^8$ cells in the loaded sample followed by a rinse with 4 mL buffer. At this condition and barring column clogging, I could run three batches of eight columns in parallel per day (a maximum that we considered feasible for control by a single, highly-trained operator) resulting in the total sorting speed of $6 \times 10^9$ cells in about 7 hours per day, at $\sim 8.3 \times 10^8$ cells recovered in the positive fraction. This also included sample preparation and deoxygenation time, and the equipment set-up time. I have concluded that more than 2,000 days are required for one person to produce $2 \times 10^{12}$ RBCs, one unit of RBC concentrate used for clinical applications by the MACS separation method, clearly an unrealistically long time.

5.5 Conclusions

RBC cultures from CCT were analyzed by CTV and separated by MACS separation system. The enriched RBCs contained Hb concentration comparable to that of the donor control RBC’s but were bigger than the donor-derived RBCs. This provided an important feedback for the HSC culture manufacturer, involved in this project, and resulted in the HSC culture condition adjustments (a verbal information from the CCT). Based on the current separation throughput, I have concluded that it would not be
practical to produce the vast numbers of cultured RBCs necessary for practical, clinical applications by the current, commercial MACS separation system. A continuous magnetic cell separation system integrated with deoxygenation process would be a promising option to scale up the process. The comparison of the cell analysis results by the CTV with other, well established methods, including Coulter counter, DIC microscopy, and RBC deformability, further confirmed the accuracy of the CTV method to single cell magnetophoresis analysis.
CHAPTER 6

LABEL-LESS MAGNETIC SEPARATION OF HUMAN SPERMATOZOA

6.1 Introduction

The application of assisted reproductive technologies (ART) to treatment of infertile couples has increased rapidly over the recent years, however the current success rate of these procedures remains suboptimal (Sunderam et al. 2009). One potential reason may be the presence of apoptotic sperm cells during in vitro fertilization (IVF) (Henkel et al. 2004; Seli et al. 2004). Apoptotic spermatozoa with impaired integrity of their cell membrane are very frequent in infertile men despite of other sperm parameters being normal (Taylor et al. 2004). The percentage of apoptotic spermatozoa increases significantly after cryopreservation (Glander and Schaller 1999), the most commonly accepted method of preserving male reproductive capacity. Therefore, selection of vital, nonapoptotic spermatozoa from fresh or cryopreserved semen samples is an important prerequisite for achieving optimal conception rates following ART.
Externalization of phosphatidylserine (PS) molecule, which is normally located in the inner leaflet of the plasma membrane of spermatozoa, is an early marker for apoptosis (Vermes et al. 1995). Thus, the PS molecules become accessible to extracellular ligands in apoptotic (due to PS externalization) and dead spermatozoa (due to the disruption of membrane integrity). Annexin V has a high affinity to PS but can not pass through the intact sperm membranes (Van Heerde et al. 1995). Magnetically-activated cell sorting (MACS) using annexin V conjugated superparamagnetic nanoparticles can effectively isolate nonapoptotic spermatozoa from apoptotic and dead ones with externalized PS molecules. In addition, there are other components in the ejaculate such as immature sperm cells, leukocytes, plasma and so on that should be removed as well prior to ART. It was reported that a combination of MACS with density gradient centrifugation (DGC) provided higher quality of spermatozoa in terms of motility, viability, and apoptosis markers compared with other conventional sperm preparation methods (Said et al. 2005).

Although the MACS system (a combination of separation columns and magnetic nanoparticles) did not exert any detectable, harmful effect on the spermatozoa (Grunewald et al. 2001), it has not yet achieved approval for clinical use. This may be due to the possibility of a small number of magnetic beads contaminating the separated sperm cell fraction for ART due to low-level binding to the spermatozoa in a very early phase of PS translocation or a weak binding to non-apoptotic cells non-specifically, resulting in insufficient cell retention in the magnetic column. Thus, the MACS separation procedure carries a potential risk of transferring the contaminating, very small magnetic beads (<100 nm) with the isolated nonapoptotic sperm cell fraction into the egg during ART. The Cleveland Clinic maintains an active clinical research ART program in
the Department of Andrology (Agarwal et al. 2008a; Agarwal et al. 2008b; Agarwal et al. 2006; Agarwal and Saleh 2002; Makker et al. 2008). The program includes evaluation of MACS methods for improving sperm cell preparation quality in application to the ART. The major concern is the risk of the magnetic bead contamination, which would preclude the use of MACS methods to ART in humans. To investigate this possibility, in collaboration with the Department of Andrology of the Cleveland Clinic, we have measured the non-specific binding of the magnetic nanobeads to the separated, viable (non-apoptotic) sperm cell fraction by measuring amount of iron in the cell fraction as compared to baseling (unlabeled sample). The bulk iron concentration was measured by inductively coupled plasma mass spectrometry (ICP-MS) in the unsorted and sorted cell fractions. The individual cell iron concentration was determined by measuring its effect on cell magnetophoretic mobility (MM) using cell tracking velocimetry (CTV). The magnitude of a cell magnetophoresis is related to the concentration of high-spin iron species in the cell (Zborowski et al. 2002). As described in the following sections, the possibility of the magnetic bead contamination of the separated sperm cells could not be excluded. An unexpected finding of this study was the evidence of a lower intracellular concentration of high-spin species following MACS separation as compared to control (the initial, unlabeled and unselected sperm cell suspension). This suggests the possibility of magnetic separation of the sperm cells due to their natural, intrinsic magnetization and prompted us to analyze the magnetic sperm cell separation process in greater detail.

Based on my preliminary data and results of a literature review, summarized at Chapter 1 and the end of this Chapter, I proposed a hypothesis that the apoptotic spermatozoa have a higher magnetic susceptibility as compared to the healthy, motile
cells because of the elevated intracellular iron and manganese (Mn). We have also considered possibility of the increased cell magnetic moment due to the known, increased level of the reactive oxygen species (ROS) content in the apoptotic sperm cells (Agarwal et al. 1994; Agarwal et al. 2008b). The ROS carry an unpaired electron spin that is detectable by electron spin paramagnetic resonance (EPR) technique (Kuppusamy 2004). Although short lived, the high concentration of ROS in the apoptotic sperm cells may contribute significantly to the cell MM when averaged over time of the magnetic separation or the CTV measurement. The observed effect could be the foundation of apoptotic sperm cells elimination without use of the magnetic labelling, based on the intrinsic cell magnetic susceptibility.

In this study, sperm samples were provided by Department of Andrology, Glickman Urological Institute, Cleveland Clinic, obtained from volunteer donors according to a protocol approved by the Institutional Review Board (IRB) research ethics committee. Prior to labelling with the annexin V magnetic beads, the cells were fractionated by density gradient centrifugation. The magnetic separation performance was evaluated in terms of cell recovery, motility, magnetophoretic mobility, and iron content in the unsorted and sorted fractions and compared with various control samples.

6.2 Materials and methods

6.2.1 Human sperm sample preparation

Fourteen human semen samples were donated for research by healthy donors of the Glickman Urological Institute at Cleveland Clinic (CCF) who signed informed consent form, approved by the Institutional Review Board of Cleveland Clinic. A silane-
coated silica particle colloid solution (PureCeption®, Sage BioPharma, Bedminster, NJ) was used for density gradient centrifugation to enrich for the predominantly mature spermatozoa. In brief, the liquefied semen was loaded onto a 40% and 80% discontinuous gradient and centrifuged at 300 g for 20 min at room temperature. The resulting 80% pellet representing mature sperm cell fraction was washed and resuspended in a modified human tubal fluid medium (QUINN’s Sperm Washing Medium, Cat. No. ART-1012, SAGE In-Vitro Fertilization Inc., Trumbull, CT). Sperm cells were classified as immotile and motile by visual inspection with a microscope, and were manually counted using CELL-UV® sperm counting chambers (Cat. No. DRM-600, Millennium Sciences, Inc., New York). The percent cell motility was reported as the percent fraction of motile cells in the sample. Subsequently, the sperm cell suspension was divided into three aliquots. The first was labeled with annexin V-conjugated microbeads and then subjected to MACS. The second was subjected to MACS directly, without incubation with magnetic beads. The last aliquot served as a control (unmanipulated). Sperm motility and cell concentration measurements were repeated in all fractions at each step of the experiment. The overall experiment procedure is illustrated in Figure 6.1.
6.2.2 MACS isolation of apoptotic spermatozoa from the labeled samples

After density gradient centrifugation, one aliquot of spermatozoa were incubated with 100 µL Annexin V microbeads (ANMB, Cat. No.130-090-201, Miltenyi Biotec, Bergisch Gladbach, Germany) at room temperature for 15 min, and then loaded in a MACS® MS column (Cat. No.130-042-201, Miltenyi Biotec, Bergisch Gladbach, Germany), which was placed in a MiniMACS™ separator magnet from Miltenyi Biotec. The spermatozoa retained in the separation column were labeled as “ANMB positive” (putative apoptotic spermatozoa bound to the magnetic particles), whereas the unretained spermatozoa were labeled as “ANMB negative” (putative non-apoptotic spermatozoa not bound to the magnetic particles), Figure 6.1. Subsequently, the ANMB positive spermatozoa were eluted from the MS column after its removal from the magnet.
6.2.3 MACS isolation of apoptotic spermatozoa from the unlabeled samples

After density gradient centrifugation, one aliquot of spermatozoa were loaded directly in a MACS® MS column placed in the MiniMACSTM magnet (without prior incubation with Annexin V microbeads). Spermatozoa retained in the separation column were labeled as “no-beads” positive, which are expected to be (intrinsically) paramagnetic. The spermatozoa eluting from the column while on the magnet are expected to be non-magnetic, and were labeled “no-beads” negative. Subsequently, the “no-beads” positive spermatozoa were eluted from the MS column after its removal from the magnet.

6.2.4 Cell analysis

For each semen sample, a total of five fractions (control, ANMB negative, ANMB positive, “no-beads” negative, “no-beads” positive) were collected and suspended in a special buffer (4.8% (v/v) 1N HCl + 95.2% (v/v) 0.9% NaCl) for cell immobilization to suppress their biological motility that could interfere with the CTV measurements. After cell number concentration determination, two aliquots for each fraction were prepared. One aliquot was diluted to a concentration of 2 - 3 × 10⁵ cells/mL for magnetophoretic mobility and sedimentation velocity analysis by CTV. Another one was digested by adding equal volume of concentrated hydrochloric acid (HCl, 36 - 38% w/w, Trace Metal Grade, Cat. No. A508-500, Fisher Scientific, Pittsburgh, PA). Digested samples were diluted 10-fold by distilled water and shipped for ICP-MS analysis at the Ohio State University (see below).
6.2.5 **CTV analysis**

A detailed description of cell magnetophoretic mobility analysis by CTV has been discussed in Chapter 1 and the protocol of cell tracking has been discussed in Chapter 3 and 4. Typically, 10 sets of images were taken for each sperm cell sample and analyzed by an imaging processing software developed in our group. Magnetophoretic mobility and sedimentation velocity distribution of over 1,000 cells in one sample were then generated.

6.2.6 **Inductively coupled plasma mass spectrometry**

A Thermo Finnigan™ Element 2 Inductively Coupled Plasma Sector Field Mass Spectrometer (ICP-MS, Thermo Electron Corporation, Bremen, Germany) in medium resolution mode (R = 4,000) was used to quantitatively evaluate the paramagnetic metal element in cells. It was performed by Mr. Anthony Lutton at the Trace Element Research Laboratory, The Ohio State University. Two elements, Fe and Mn were quantified in each sample. Fe was selected because it is a major component of the magnetic microbeads and therefore a good indicator of a potential microbeads contamination in the ANMB negative cell fraction. Mn was selected as a likely major contributor to the natural (intrinsinc) cell paramagnetism (along with Fe)

6.2.7 **Statistical analysis**

Wilcoxon signed-rank test was used to test for differences between samples. Spearman’s rank order correlation coefficient between sperm parameters was calculated and tested against null hypothesis. P < 0.05 was considered statistically significant. The
statistical analysis was performed using SigmaStat 3.5 (Systat Software, Inc., Chicago, IL).

6.3 Results and discussion

6.3.1 Comparison of cell recovery and motility between labeled and unlabeled samples after MACS separation

Two fractions, ANMB negative and ANMB positive, were separated from the labeled sperm samples after MACS separation (Figure 6.1). In a similar manner, two fractions, “no-beads” negative and “no-beads” positive, were isolated from the unlabeled sample that was also subjected to MACS separation, Figure 6.1. Sperm cell motility in all four fractions after MACS separation was examined and compared in Figure 6.2 and Table 6.1. The motility of cell fractions from first 4 semen samples was not analyzed for technical reasons. Overall, for both labeled and unlabeled samples, the cell motility in negative fraction after separation is significantly higher than that in the positive fraction (labeled samples: median 71.0% vs. 9.0%, P = 0.002, n = 10; unlabeled samples: median 77.5% vs. 11.0%, P = 0.002, n = 10). This indicates that low motility cells, labeled or unlabeled, were eliminated by MACS separation. The result is highly significant but wholly unexpected, and prompted my additional investigations into the possibility of the intrinsic cell magnetophoresis mechanism of the observed phenomenon. There is no significant difference in motility between ANMB negative and “no-beads” negative (P = 0.28, n = 10), and between ANMB-positive and “no-beads” positive samples (P = 0.49, n = 10). In the labeled samples, only apoptotic or dead spermatozoa were bound with magnetic beads as a result of PS externalization and captured by MACS columns. It was
reported by others that there is a significant inverse correlation between sperm motility and apoptosis in ejaculated sperms (Barroso et al. 2000; Chen et al. 2006; Oosterhuis et al. 2000; Shen et al. 2002). Therefore, it is likely that the positive cells from the unlabeled samples captured by MACS columns are also apoptotic because their motility is lower than that of the cells in the negative fraction, and comparable to that of the ANMB positive cell fraction, Figure 6.2.

Figure 6.2 Comparison of motility between labeled and unlabeled samples after MACS separation. Each open circle in each group represents the motility data of one sample (n = 10). The boundaries of the box closest to zero and farthest from zero indicate the 25th and 75th percentiles; whiskers (error bars) above and below the box indicate the 90th and 10th percentiles; a line within the box marks the median.
Comparison of cell number in ANMB negative and “no-beads” negative fractions, and in ANMB positive and “no-beads” positive fractions is shown in Table 6.1 and Figure 6.3. Cell number collected in the negative fractions after separation is significantly higher than that in the positive fractions (P < 0.001, n = 14) as expected if the assumed mechanism of the intrinsic magnetophoresis of the apoptotic cells is indeed correct, because semen samples from healthy donors contain very few apoptotic cells. Most interestingly, no significant differences were observed between the respective pairs of negative and positive fractions (ANMB negative vs. “no-beads” negative: P = 0.17, n = 14; ANMB positive vs. “no-beads positive”: P = 0.13, n = 14). These results strongly
suggest that similar number of cells in the unlabeled samples were retained in the MACS column as in the labeled samples. In other words, the data suggest that the magnetic labeling with annexin V magnetic beads was not necessary for depletion of the apoptotic cells.

Figure 6.3  Comparison of the recovered cell number between labeled and unlabeled samples after MACS separation. Each open circle in each group represents the cell # data of one sample (n = 14). The boundaries of the box closest to zero and farthest from zero indicate the 25th and 75th percentiles; whiskers (error bars) above and below the box indicate the 90th and 10th percentiles; a line within the box marks the median.
6.3.2 Comparison of % fraction of cells with cut-off MM > $1.5 \times 10^{-5}$ mm$^3$/T.A.s before and after separation

Magnetophoretic mobility of sperm cells before and after MACS separation was measured by CTV. In nature, most cells are diamagnetic because of the diamagnetic properties of their major components, such as water, proteins, nucleic acids, carbohydrates, and lipids (Chikov et al. 1991; Torbet 1987). Therefore, the expected MM value of sperm cells is negative or very close to zero. Based on the work on the magnetophoretic mobility of red blood cells (RBCs), described in Chapter 5, the expected sperm cell MM values are within the range of $\pm 10^{-5}$ mm$^3$/T.A.s. An example of such a distribution is shown in Figure 6.4.

![Figure 6.4 MM distribution of one sperm cell sample.](image)
However, I have consistently observed MM of sperm cells significantly greater than the upper limit of that range. In fact, 0.1 - 1.6% of cells in 12 of 14 control samples exhibited MM that was higher than the $1.5 \times 10^{-5} \text{mm}^3/\text{TAs}$. Visual inspection of the MM distribution of those samples indicates that $1.5 \times 10^{-5} \text{mm}^3/\text{TAs}$ is a reasonable “gate”, above which a cell can be considered “magnetic”, Figure 6.5. By that criterion, the percentage of “magnetic” cells (with MM $> 1.5 \times 10^{-5} \text{mm}^3/\text{TAs}$) in control samples is significantly greater than that in ANMB negative fractions obtained from the ANMB labeled samples ($P = 0.006$, $n = 14$, one-tailed), but only slightly greater than that in “no-beads” negative fractions from unlabeled samples ($P = 0.05$, $n = 14$, one-tailed), Figure 6.6 (A, B). There is no significant difference between “no-beads” negative and ANMB negative fraction ($P = 0.83$, $n = 14$, two-tailed), Figure 6.6 (C). This suggests that MACS separation could deplete not only the apoptotic cells labeled with magnetic beads, but also additional “magnetic” sperm cells that did not require labeling for separation. A similar effect was observed following separation of the unlabeled sample. It is likely that these “magnetic”, unlabeled cells were cells with high intrinsic magnetic susceptibility that contributed to the higher than expected cell depletion in the ANMB labeled sample, and were responsible for the magnetic cell depletion effect in the unlabeled cell sample.
Figure 6.5  Dot plots of MM against settling velocity of control, “no-beads” negative fraction from sample #2.

Continued
Figure 6.6 Comparison of % fraction of cells with MM > 1.5e-5 mm³/TAs in samples before and after MACS separation, (A) control vs. “no-beads” negative fraction; (B) control vs. ANMB negative fraction; (C) “no-beads” negative vs. ANMB negative fractions.
<table>
<thead>
<tr>
<th></th>
<th>% fraction of “magnetic” cells (MM &gt; 1.5 × 10^{-5} mm^3/TAs)</th>
<th>Fe content (fg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANMB negative (A)</td>
<td>0.089 (0.00, 0.17)</td>
<td>8.38 (3.59, 18.00)</td>
</tr>
<tr>
<td>“No-beads” negative (C)</td>
<td>0.088 (0.082, 0.18)</td>
<td>3.90 (3.39, 6.00)</td>
</tr>
<tr>
<td>Control</td>
<td>0.21 (0.14, 0.62)</td>
<td>7.93 (4.91, 22.50)</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>control vs. A</td>
<td>0.006 (one-tailed)</td>
<td>1.0 (two-tailed)</td>
</tr>
<tr>
<td>control vs. C</td>
<td>0.05 (one-tailed)</td>
<td>0.0085 (one-tailed)</td>
</tr>
<tr>
<td>A vs. C</td>
<td>0.83 (two-tailed)</td>
<td>0.007 (two-tailed)</td>
</tr>
</tbody>
</table>

Table 6.2 Descriptive statistics of % fraction of “magnetic” cells and mean Fe content per cell after MACS separation presented as median (25\textsuperscript{th}, 75\textsuperscript{th} percentiles). n = 14; P < 0.05.

6.3.3 Comparison of Fe content before and after MACS separation

The Fe and Mn content in each sample (controls, ANMB negatives, and “no-beads” negatives) were analyzed by ICP-MS. The mean Fe content per cell in control samples is significantly greater than that in “no-beads” negative fraction (P = 0.0085, n = 14, one-tailed), Figure 6.7 (A). Based on cell number and Fe mass balance before and after separation, therefore, the mean Fe content per cell in “no-beads” positive fraction with low motility is expected to be higher than that in unsorted control. This agrees with results obtained from CTV measurements, discussed above. The iron atoms in the “no-beads” positive fraction are likely in a high spin state resulting in a relatively high magnetic susceptibility of the cells captured on the magnet. As discussed below,
excessive concentration of “free” iron plays an important role in the formation of intracellular ROS. The elevated ROS production has been associated with decreased sperm motility and increased DNA damage and apoptosis (Agarwal et al. 1994; Agarwal et al. 2008b; Aziz et al. 2004; Griveau and Le Lannou 1997; Wang et al. 2003). Due to the uncompensated electron spin, ROS are also paramagnetic molecules.

Interestingly, no significant differences between control and ANMB negative cell fractions were found (P = 1.0, n = 14, two-tailed), Figure 6.7 (B). This seems to contradict results from CTV analysis, discussed above. However, it is likely that the discrepancy comes from the fact that ICP-MS is less sensitive to cell iron content than CTV, because it determines the average Fe content in a sample, whereas CTV measures MM of each single cell in the sample. Two samples with the same number of cells may contain the same total Fe concentration. But this does not preclude a situation in which the fraction of cells with relatively high Fe content (reflected by their high MM detected by CTV) in these two samples could be different. ANMB negative fraction contains fewer “magnetic” cells (MM > 1.5 × 10^{-5} \text{mm}^3/TAs) than that in control. But the “magnetic” cells in the negative fraction may possess more iron atoms than the “magnetic” cells in control. This suggests that the presence of the “magnetic” cells in ANMB negative fraction are the result of contamination by the magnetic beads. As stated earlier, it is likely that a limited number of magnetic beads binds to spermatozoa in a very early phase of PS translocation or binds non-specifically to non-apoptotic cells, with the resulting low magnetization of those cells and a lack of their retention in the column. Such low level magnetic bead binding introduced iron and resulted in high MM in a number of cells collected in ANMB negative fraction. It is also possible that these
magnetic beads are freely suspended in the ANMB negative fraction media, and therefore are too small to be detected by CTV. However, they are detectable by ICP-MS. All things considered, our primary concern about the possibility of transferring the contaminating magnetic nanobeads (<100 nm) with the magnetically isolated, nonapoptotic sperm cell fraction into the egg during ART cannot be rejected based on the data from my studies.

Continued

Figure 6.7 Comparison of Fe content in samples before and after MACS separation. (A) control vs. "no-beads" negative fractions; (B) control vs. ANMB negative fractions
Compared to the intracellular Fe, much lower intracellular Mn concentration was measured in each sample (on average, about 100-fold lower). Hence, the contribution of Mn to the magnetophoresis was neglected.

### 6.3.4 Correlation between Fe content and motility

In the course of these studies I have developed a hypothesis that the apoptotic, low motility spermatozoa possess relatively high Fe concentration. Therefore, a negative correlation between Fe concentration and cell motility is expected. This was confirmed by the negative correlation coefficients obtained for Spearman’s rank order correlation between those two parameters, Table 6.3. However, no such significant relationship between intracellular Fe concentration and cell motility was observed for either “no-beads” negative fraction (Figure 6.8 A) or ANMB negative fractions (Figure 6.8 B).
Insufficient statistical power to detect such difference, related to the limited number of sperm cell samples available for this study may account for lack of achieving statistical significance. In ANMB negative fractions, the contamination by the magnetic beads due to labeling are a likely cause for the intracellular iron concentration in these samples to be overestimated.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Motility (%)</th>
<th>Correlation coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe content (fg/cell)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANMB negative</td>
<td></td>
<td>-0.382</td>
<td>0.258</td>
</tr>
<tr>
<td>“No-beads” negative</td>
<td></td>
<td>-0.503</td>
<td>0.126</td>
</tr>
</tbody>
</table>

Table 6.3  Spearman’s rank order correlation between motility and Fe content at P < 0.05.
Figure 6.8  Relationship between motility and Fe content in “no-beads” negative (A) and in ANMB negative (B) fractions.
6.4 Conclusions

MACS is considered a feasible, fast, and simple cell sorting method, which is widely used to cell separation according to specific cell surface markers. The combination of MACS with DGC efficiently selected a sperm population with high motility by using annexin V-conjugated microbeads that bind to externalized PS in apoptotic or dead cells. In this study, the possibility of magnetic beads contamination in the selected cell population was confirmed in a clinically important model of human sperm cell separation. No difference in the Fe concentration between control and ANMB negative fractions was observed. However, the fraction of cells with \( \text{MM} > 1.5 \times 10^5 \, \text{mm}^3/\text{TAs} \) in the control sample was significantly greater than that in ANMB negative fraction. These two independent results indicate possible contamination by the magnetic beads of the magnetically separated spermatozoa population, which could be bound to cells in too low numbers for these cells to be depleted on the magnet, or suspended freely in the solution.

The experimental data showed evidence of magnetic sperm cell separation without magnetic bead labeling. Based on the hypothesis that apoptotic spermatozoa have a higher magnetic susceptibility than that of the healthy, motile cells, a combination of label-less MACS and DGC was then investigated and compared with the magnetic bead labeling method.

I have found that MACS separation worked well on unlabeled samples. There was no difference in corresponding, separated fractions between labeled and unlabeled samples in terms of cell recovery and cell motility after separation. For both labeled and unlabeled samples, the cell motility in negative fraction was greater than that in the positive fraction,
indicating that the low motility cells, whether labeled or unlabeled, were eliminated by MACS separation.

The Fe concentration and % fraction of the “magnetic” cells in the control sample was significantly greater than those in the “no-beads” negative fraction. This demonstrates that cells with higher magnetic susceptibility (presumably due to their higher intracellular iron and or ROS concentration, see below) were depleted by MACS separation, in agreement with my hypothesis.

High intrinsic magnetic susceptibility caused by elevated paramagnetic metal concentration, such as iron ions, or ROS concentration may have contributed to the sperm cell separation results and high MM observed in cells. ROS are free radicals, which are paramagnetic molecules containing one or more electrons with unpaired spin. Uncontrolled and excessive production of ROS causes lipid peroxidation of the sperm plasma membrane, decreased sperm concentration and motility, DNA strand breakage, and sperm cell apoptosis (Agarwal et al. 2008b; Ollero et al. 2001). Damage induced by ROS has been identified as a major factor in male infertility (Agarwal et al. 2003; Sharma and Agarwal 1996; Sikka 2001). On the other hand, excessive “free” iron (Fe$^{2+}$ or Fe$^{3+}$) which is in high-spin state with four or five unpaired electrons catalyzes the formation of ROS through Fenton reaction (Fenton 1894; Jacob et al. 1997; Toyokuni 1996) and Haber-Weiss reaction (Halliwell and Gutteridge 1992). Additionally, manganese is another paramagnetic essential metal present in cells, which mainly functions as a constituent of and active oxygen scavenger, manganese-superoxide dismutase (MnSOD) (Keen et al. 2000). Increased MnSOD expression has been observed in some cancer tissues to respond to intrinsic ROS stress (Hileman et al. 2004; Hu et al. 2005). These are
mechanisms to consider in the future work on the magnetic cell separation based on intrinsic magnetic cell susceptibility.

Due to the limited sample size, I was not able to detect if there was statistical significance in the negative correlation between motility and Fe concentration in either “no-beads” negative or ANMB negative fractions. Overestimation of the intracellular Fe content in ANMB negative fraction caused by the contamination of magnetic beads may have also masked the relationship between those two cell parameters.

In summary, a label-less magnetic separation of nonapoptotic spermatozoa relying on the high intrinsic magnetic susceptibility of apoptotic cells alone is highly desirable and appears to be feasible based on my studies. However, more work has to be done before such a method could be put to a practical use, such as the measurement of ROS contribution to the magnetic susceptibility, and the magnetophoretic mobility, of the apoptotic sperm cells.
CHAPTER 7

INTRINSIC MAGNETOPHORESIS OF CANCER CELLS

7.1 Introduction

Magnetic cell separation and detection has been widely employed in the area of biological research and selected clinical applications (Zborowski and Chalmers 2008). In principle, it is driven by the difference in magnetophoretic mobility between different cell subsets as a result of the difference in their relative magnetic susceptibilities in aqueous media (Zborowski et al. 2002). In nature most cells are diamagnetic with a negligible magnetophoretic mobility. In order to increase the difference in cell magnetophoresis, one increases the magnetic susceptibility of target cells by binding of magnetizable micro- or nano-particles to the cell surface through antigen-antibody reaction or other suitable receptor-ligand reaction. Therefore, the specificity of the separation and detection highly relies on the cell immunophenotype and the properties of antibodies that distinguish between characteristic cell surface antigen markers. In practical applications, the magnetic cell separation and detection apparatus is low-cost and the separation process is typically simple and fast. The limiting factor is the high cost of the
immunomagnetic reagents. In addition, the labeling process complicates the sample preparation, which is time consuming and labor intensive and adds significantly to the overall cost of the magnetic separation.

A magnetic field-based cell separation and detection method relying solely on the intrinsic magnetic susceptibility of cells is an attractive alternative because it frees the process from the expensive and laborious immunomagnetic labeling steps and the cost of reagents. A label-free magnetic cell separation has been proposed in the past. Notable examples include ferromagnetic behavior of certain prokaryotic cells as a result of the synthesis of magnetosomes (Bazylinski and Frankel 2004; Bazylinski et al. 1994). Deoxygenated or methemoglobinated erythrocytes (deoxy RBC or metHb RBC), discussed in Chapter 5, as well as malaria infected RBCs, are paramagnetic relative to the aqueous physiologic electrolyte solution because of the existence of unpaired electrons in the four heme groups or of hemozoin (Bohle et al. 1998; Moore et al. 2006). New applications were evaluated in the course of this thesis work. As described in Chapter 5, a post-differentiation magnetic separation method was used to enrich the cultured RBCs from hematopoietic stem cell cultures by exploiting the paramagnetic nature of deoxy RBCs and metHb RBCs (relative to the suspending aqueous medium). In Chapter 6, I proposed that apoptotic spermatozoa possess higher intrinsic magnetic susceptibility than the non-apoptotic ones caused by elevated iron and reactive oxygen species (ROS) content and then developed a label-less magnetic depletion process of apoptotic spermatozoa simply relying on their high intrinsic magnetic susceptibility. The experimental results suggest the method to be feasible and promising.
In this chapter, I describe my studies on the possibility of extending this label-less, intrinsic magnetic cell susceptibility-based approach to certain cancer cells. Cancer cells are likely to have significant intrinsic paramagnetic susceptibility as discussed in Chapter 1. The proposition that the altered electronic structure of key metabolic compounds is related to cell disease state, including malignant transformation, has been discussed before (Szent-Györgyi 1976). It was reported that iron overload could lead to neoplastic transformation. Rapidly dividing cancer cells have a higher requirement for iron than normal cells, resulting in an increased expression of proteins important for iron transfer into the cell, such as transferrin receptor 1 (TfR1) (Larrick and Cresswell 1979; Weinberg 1992). It was also found that iron overload disrupts the redox balance of the cell and generates excessive ROS, which modulates signaling networks related to malignant transformation (Benhar et al. 2002; De Freitas and Meneghini 2001; Papanikolaou and Pantopoulos 2005). Another paramagnetic metal element, manganese, may also play an important role in certain cancers as described in Chapter 1.

Although the existence of cancer cells with high magnetic susceptibility is plausible, as discussed above, the technology for label-less magnetic cell separation and detection is largely non-existent. In my work, I have relied on unique capabilities of the cell magnetophoretic mobility analyzer, developed in the laboratories of Drs. Chalmers and Zborowski. Cell tracking velocimetry (CTV), an experimental instrument developed to measure cell magnetophoretic mobility (MM) is utilized in this study to determine magnetic properties of various cancer cells. Magnetophoretic mobility is a measure of cell intrinsic or imparted magnetophoresis induced by an imposed magnetic field in a viscous medium (It is analogous to cell electrophoretic mobility in aqueous media.
exposed to the electric field, or cell dielectrophoretic mobility when the imposed electric field is oscillating (Markx and Pethig 1995; Watarai et al. 1997)). Established human cancer cell lines are routinely used as experimental models for human cancers (Masters 2000). Here, magnetophoresis of eight cancer cells lines were measured. Moreover, paramagnetic metal elements, Fe and Mn in cells were quantitatively evaluated by ICP-MS. Oxy RBC was selected as a negative control and metHb RBC was selected as a positive control for MM study. Additionally, the expression of TfR1 was quantified and label-less magnetic separations were performed on selected cancer cell lines. Such studies may be of vital importance for potential future applications in clinical diagnosis of certain cancers.

7.2 Materials and methods

7.2.1 Cancer cell lines

Table 7.1 lists all the cancer cell lines used in this study. The cell preparations were obtained from ATCC (The American Type Culture Collection, Manassas, VA) or the Cleveland Clinic (CCF, Cleveland, OH). These cells were cultured in 75 cm² T-flasks (BD Bioscience, Bedford, MA) in the complete media (the formulation of the used media is listed in Appendix B), and the complete media with iron compound addition. The cell cultures were maintained at 37°C and 5% CO₂ and passaged every two or three days using sterile technique to sustain viability. Up to 20 cell passages were used. Adherent cells were detached by trypsin-EDTA solution (0.05% trypsin, 0.53 mM EDTA, product no. 521-100, Central Cell Services, CCF, Cleveland, OH). The two iron compounds, Fe(NO₃)₃ (Cat. No. F8505) and ferric ammonium citrate (FAC, Cat. No. F5879-100G),
were purchased from Sigma-Aldrich, St Louis, MO. Final iron concentrations in media were adjusted as specified for each experiment.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>ATCC No.</th>
<th>Description</th>
<th>Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAL 27</td>
<td>CRL-2095</td>
<td>tongue carcinoma</td>
<td>DMEM&lt;sup&gt;b&lt;/sup&gt; + 10% FBS&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Detroit 562</td>
<td>CCL-138</td>
<td>pharyngeal carcinoma</td>
<td>AMEM&lt;sup&gt;c&lt;/sup&gt; + 10% FBS&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HeLa&lt;sup&gt;a&lt;/sup&gt;</td>
<td>CCL-2</td>
<td>Epitheloid carcinoma</td>
<td>RPMI1640&lt;sup&gt;b&lt;/sup&gt; + 10% FBS&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hep 3B 2.1-7</td>
<td>HB-8064</td>
<td>hepatocellular carcinoma</td>
<td>EMEM&lt;sup&gt;d&lt;/sup&gt; + 10% FBS&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hep G2</td>
<td>HB-8065</td>
<td>hepatocellular carcinoma</td>
<td>EMEM&lt;sup&gt;d&lt;/sup&gt; + 10% FBS&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HL-60</td>
<td>CCL-240</td>
<td>promyelocytic leukemia</td>
<td>IMDM&lt;sup&gt;d&lt;/sup&gt; + 20% FBS&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>K-562</td>
<td>CCL-243</td>
<td>chronic myelogenous leukemia</td>
<td>IMDM&lt;sup&gt;d&lt;/sup&gt; + 10% FBS&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>U-937</td>
<td>CRL-1593.2</td>
<td>histiocytic lymphoma</td>
<td>RPMI1640&lt;sup&gt;d&lt;/sup&gt; + 10% FBS&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

(IMDM: Iscove’s Modified Dulbecco’s Medium; DMEM: Dulbecco’s Modified Eagle’s Medium; AMEM: Alpha Modification of Eagle’s Medium; EMEM: Eagle’s Minimum Essential Medium; FBS: Fetal Bovine serum; <sup>a</sup> this cell line was a gift from Carmel Burns, Central Cell Services, CCF; <sup>b</sup> from Central Cell Services, CCF; <sup>c</sup> from Mediatech, Inc., Manassas, VA ); <sup>d</sup> from ATCC. See Appendix B for media composition.

Table 7.1 Detailed information about cancer cell lines used in this study.

Cell size and concentration were evaluated by a Z2™ Coulter Counter® (Beckman Coulter Inc., Fullerton, CA, USA) equipped with a 70 µm aperture. The cell viability was determined by trypan blue (Cat. No. 15250-061, Invitrogen, Grand Island, NY) exclusion method.
7.2.2 Determination of TfR1 (CD71) expression

TfR1 (CD71) is involved in the cellular uptake of iron and is expressed on cells with high proliferative activity such as cancer cells (Larrick and Cresswell 1979; Weinberg 1992). An immunofluorescent labeling protocol was used to target TfR1 expressing cells, as summarized here. The cell suspension collected from T-flask was washed by a degassed labeling buffer containing 1× PBS, 2 mM EDTA, and 0.5% BSA. Thirty µL/10^6 cells of primary antibody, anti-human CD71-PE (Cat. No.555537, BD Biosciences, San Jose, CA) was then added to the cell pellet. After 30 minutes of incubation on ice, cells were washed once with the labelling buffer and resuspended in 0.4 mL PBS for flow cytometry analysis.

The cell fluorescence analyses were performed in the Flow Cytometry Core (CCF, Cleveland, OH). QuantiBRITE™ PE beads (Cat. No. 340495, BD Biosciences, San Jose, CA) were used to estimate the number of antibodies (anti-CD71-PE) bound per cell.

7.2.3 Enrichment of “magnetic” cells by MACS cell separation

Cells for separation were collected from the culture flasks into conical falcon tubes and washed once (i.e., by addition of labeling buffer, centrifugation at 250 g, 4°C for 10 minutes, followed by decanting of supernatant). The pellet was re-suspended in 0.5 mL degassed PBS to a cell number concentration of (2 - 8.5) × 10^7 cells/mL. The suspension was subsequently passed through a MACS® MS column (Cat. No. 130-042-201, Miltenyi Biotec, Bergisch Gladbach, Germany) which was placed in the MiniMACSTM separator. The column was washed with 1 mL PBS twice. The cells that passed through the column on the magnet and the washing effluent were collected.
together in one tube and labeled as negative fraction. Subsequently, the MS column was removed from the magnet and the cells retained in the column were flushed out with 1 mL PBS by applying the plunger supplied with the column. These cells were referred as positive fraction. After evaluation of the cell concentration and viability in all three fractions: the negative and positive cells as well as the feed (the cell sample before separation), the fractions were split into three aliquots each for CTV, ICP-MS, and cell cycle analysis.

7.2.4 Cell surface marker analysis by flow cytometry

Flow cytometric analysis was conducted in the Flow Cytometry Core (CCF, Cleveland, OH) on a fluorescence- activated cell sorter (FACS, BD LSR I, BD Biosciences, San Jose, CA, USA), and using a FACSDiva software V5.2.0. Control sample consisted of unstained cell suspension (negative control). Stained cell samples that were not analyzed immediately after labeling were fixed using a 1% \( \text{v/v} \) formaldehyde (Cat. No. 18814, Polysciences, Inc. Warrington, PA) in PBS solution for later analysis. The cells were kept in dark at 4°C after fixing till analysis on the flow cytometer. During the flow cytometry analysis, at least 10,000 cells were evaluated for each sample. The evaluation of the flow data was conducted using a freeware provided by Joe Trotter of the Scripps Institute, La Jolla, CA (WinMDI).

7.2.5 Cell cycle analysis by flow cytometry

To quantitate cellular DNA content, cells was first permeabilized by fixation with 70% ethanol at 4 °C for 30 min. Cell suspension was then centrifuged at about 200 g for 6 minutes and resuspended in PBS with a concentration of \( 1 \times 10^6 \) cells/mL. The
instructions for the rest of the protocol read as follows: add 5 µL RNase (DNase-free) to
1 mL of cell suspension and incubate at 37 °C for 30 minutes. After that, chill the cell
suspension on ice and add 100 µL propidium iodide (PI, Cat. No. 11399861001, Roche
Applied Science, Indianapolis, IN) to the cell suspension. Collect red cell fluorescence
(indicating PI binding) on a flow cytometer in the Flow Cytometry Core (CCF, Cleveland,
OH) and analyze by a commercially available software ModFit LT™ (Verity Software
House, Topsham, ME).

7.2.6 Analysis of the cell magnetophoretic mobility distribution by CTV

Before CTV analysis, cell suspension buffer was replaced with PBS containing
0.1% Pluronic F-68. For each sample, 3 mL cell suspension was prepared with a
concentration of (2 - 3) × 10^5 cells/mL. Normally, motion of over 1,000 cells in one
sample was recorded and analyzed by an imaging processing software developed in our
group. Magnetophoretic mobility and sedimentation velocity distribution in one sample
were then generated. The details of the CTV analysis are provided in Chapter 3 and
Appendix A.

7.2.7 Inductively coupled plasma mass spectrometry

A Thermo Finnigan™ Element 2 Inductively Coupled Plasma Sector Field Mass
Spectrometer (ICP-MS, Thermo Electron Corporation, Bremen, Germany) in medium
resolution (R = 4,000) was used to quantitatively evaluate the paramagnetic metal
element in cells. It was performed at the Trace Element Research Laboratory, The Ohio
State University. Before analysis, cells were digested by adding same volume of
concentrated nitric acid (HNO₃, Trace Metal Grade, Cat. No. A509-500, Fisher Scientific,
Pittsburgh, PA). Digested samples were then diluted 10-fold by distilled water. The concentration of two elements, Fe and Mn were measured in each sample.

### 7.2.8 Statistical analysis

Kruskal-Wallis test was used to compare the equality of sample medians among groups using SigmaStat 3.5 software package (Systat Software, Inc., Chicago, IL). Student’s t-test and Tukey-Kramer test were applied to compare the mean differences between samples, using JMP software (SAS Institute, Cary, NC). The confidence level of $P < 0.05$ was considered as statistically significant.

### 7.3 Results and discussion

#### 7.3.1 Magnetic susceptibility of culture media

More than 95% volume of the complete cell media is the diamagnetic water. Magnetic susceptibilities of some other media components are listed in Table 7.2, most of which are more diamagnetic than water except of the ferric nitrate. The ferric nitrate was included in DMEM medium at a very low concentration, 0.10 mg/L (Appendix B) in this study, and therefore its contribution to the bulk magnetic susceptibility of DMEM medium could be neglected ($\phi\chi = 0.10 / (1.68 \times 10^6) \times 796.15 \times 10^{-6} = 4.7 \times 10^{-11}$, 1.68 $\times 10^6$ mg/L is density of ferric nitrate).
<table>
<thead>
<tr>
<th>Substance</th>
<th>Temperature (K)</th>
<th>$\chi \times 10^6$ (SI)*</th>
<th>Concentration in normal media (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>293</td>
<td>-9.04</td>
<td>~985</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>ord.</td>
<td>-13.32</td>
<td>0 - 0.2</td>
</tr>
<tr>
<td>Ca(NO$_3$)$_2$</td>
<td>ord.</td>
<td>-8.80</td>
<td>0 - 0.1</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>294</td>
<td>-13.88</td>
<td>0.04884 - 0.09767</td>
</tr>
<tr>
<td>KCl</td>
<td>ord.</td>
<td>-13.04</td>
<td>0.33 - 0.40</td>
</tr>
<tr>
<td>KNO$_3$</td>
<td>ord.</td>
<td>-8.83</td>
<td>0 - 0.000076</td>
</tr>
<tr>
<td>NaCl</td>
<td>ord.</td>
<td>-14.11</td>
<td>4.505 - 6.800</td>
</tr>
<tr>
<td>Alanine</td>
<td>-</td>
<td>-10.11</td>
<td>0 - 0.025</td>
</tr>
<tr>
<td>Asparagine</td>
<td>288</td>
<td>-10.20</td>
<td>0 - 0.05682</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>285</td>
<td>-10.05</td>
<td>0 - 0.03</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>-</td>
<td>-9.78</td>
<td>0 - 0.075</td>
</tr>
<tr>
<td>Glycine</td>
<td>323</td>
<td>-10.63</td>
<td>0.0075 - 0.0500</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>-</td>
<td>-9.47</td>
<td>0.050 - 0.105</td>
</tr>
<tr>
<td>Leucine</td>
<td>-</td>
<td>-10.51</td>
<td>0.050 - 0.105</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>-</td>
<td>-10.88</td>
<td>0.005 - 0.016</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>-</td>
<td>-10.63</td>
<td>0.02883 - 0.10400</td>
</tr>
<tr>
<td>Valine</td>
<td>-</td>
<td>-9.80</td>
<td>0.020 - 0.094</td>
</tr>
<tr>
<td>vitamin B-12</td>
<td>-</td>
<td>-8.67</td>
<td>0 - 0.000136</td>
</tr>
<tr>
<td>D-glucose</td>
<td>298</td>
<td>-10.92</td>
<td>1.0 - 4.5</td>
</tr>
<tr>
<td>Fe(NO$_3$)$_3$·9H$_2$O</td>
<td>293</td>
<td>796.15</td>
<td>0 - 0.0001</td>
</tr>
</tbody>
</table>

*Volume magnetic susceptibilities in SI unit were calculated from mass, molar, or volume magnetic susceptibilities in CGS units provided by CRC Handbook of Chemistry and Physics (Weast 1981) using the following equations:

$$\chi \ (\text{CGS}) = \rho \chi_g \ (\text{CGS}), \ \chi \ (\text{CGS}) = \rho \chi_m \ (\text{CGS})/M_w, \ \chi \ (\text{SI}) = 4\pi\chi \ (\text{CGS})$$

Table 7.2 Volume magnetic susceptibilities of some cell media components
In addition, 10% FBS is usually added to the cell media, which contains iron as a necessary component for normal cell metabolism. Kakuta et al. (Kakuta et al. 1997) reported an average value of 2.40 mg/L iron in FBS by measuring 13 lots of commercial FBS. Assume conservatively that all these iron atoms are in high spin state with a magnetic susceptibility of $14,000 \times 10^{-9}$ L/mol (same as that in metHb RBC (Coryell et al. 1937; Hackett et al. 2009)). It follows that the total Fe magnetic susceptibility contribution in the media is $7.5 \times 10^{-10}$ ($c_{Fe} \chi_{m,Fe} = 2.4 \times 10^{-3} \times 10\% \times 4\pi \times 14,000 \times 10^{-9} / 56$, SI unit), which also can be ignored in comparison with the volume magnetic susceptibility of water, $-9.04 \times 10^{-6}$. Note that the bulk volume magnetic susceptibility of the media is the weighted sum of the susceptibilities of its components.

### 7.3.2 Characterization of cancer cells

Cells at subconfluence cultured in the complete media were collected, washed, and suspended in PBS. Cell size distributions were determined by Coulter counter. The mean cell diameters for all eight cancer cell lines are shown in Table 7.3. Two metal elements, Fe and Mn were measured by ICP-MS. Average Fe and Mn content per cell and per cell volume are also listed in Table 7.3. Two hepatocellular cells possess the highest Fe and Mn contents. This is consistent with the function of the liver as the iron storage and metabolism site. It is well known that a number of diseases of the liver, such as liver fibrosis, cirrhosis, and hepatoma are related to the accumulation of iron in the hepatocyte (Bonkovsky 1991).
### Table 7.3  Cell size and metal content

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Diameter (µm)</th>
<th>Fe fg/cell</th>
<th>Fe ng/mL</th>
<th>Mn fg/cell</th>
<th>Mn ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAL 27</td>
<td>13.7 ± 1.80</td>
<td>20.66</td>
<td>15,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Detroit 562</td>
<td>14.5 ± 1.68</td>
<td>25.26</td>
<td>16,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HeLa</td>
<td>15.8 ± 1.90</td>
<td>45.65</td>
<td>22,000</td>
<td>1.23</td>
<td>596</td>
</tr>
<tr>
<td>Hep 3B 2.1-7</td>
<td>15.5 ± 2.31</td>
<td>96.15</td>
<td>49,000</td>
<td>5.70</td>
<td>2923</td>
</tr>
<tr>
<td>Hep G2</td>
<td>14.8 ± 2.55</td>
<td>57.93</td>
<td>34,000</td>
<td>6.86</td>
<td>4041</td>
</tr>
<tr>
<td>HL-60</td>
<td>12.1 ± 1.53</td>
<td>17.12</td>
<td>18,000</td>
<td>0.82</td>
<td>884</td>
</tr>
<tr>
<td>K-562</td>
<td>16.4 ± 2.07</td>
<td>47.54</td>
<td>21,000</td>
<td>0.93</td>
<td>403</td>
</tr>
<tr>
<td>U-937</td>
<td>13.2 ± 1.37</td>
<td>0.57</td>
<td>473</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

#### 7.3.3  MM of cells incubated in the complete media

The complete cell media information used in this study is provided in Table 7.1 and Appendix B. After inoculation, cell samples were taken every 12 to 24 hours for cell counting, viability determination, and MM measurement by CTV. Before CTV analysis, cells were washed once with PBS containing 0.1% Pluronic F-68 rather than the PBS buffer containing 0.5% BSA and 2 mM EDTA that is a common buffer used for magnetic cell separation. The use of Pluronic in the buffer was suggested by the following reasons. The EDTA is added to the buffer to bind free divalent ions (Ca²⁺, Mg²⁺) in the sample and minimize cell adhesion to surfaces and to each other. This helps to avoid cell losses from the sample and cell clumping. The BSA works as a stabilizing agent to help prevent non-specific cell adhesion. But BSA may be contaminated by iron during its production process. Moreover, the EDTA can form strong complexes with Fe (III). The presence of
iron in this formulation of the cell suspension buffer may therefore affect the MM measurement by CTV. Consequently, we have replaced EDTA and BSA with 0.1% Pluronic-F68. The use of Pluronic for work with cell cultures has been reported by others. It was found that supplementation with Pluronic during culture prevented loss of cell viability, indicating protection in either a gas sparged or bubble-free environment (Tharmalingam et al. 2008). During my experiments, no aggregation was observed by using buffer PBS + 0.1% Pluronic. The iron content in buffer PBS + 0.1% Pluronic is very close to pure PBS but lower than in the other buffer (i.e., PBS + 2 mM EDTA + 0.5% BSA), Table 7.4. Importantly, the iron content in all three buffers is much lower than that in the cancer cells, compare Table 7.3 with Table 7.4.

<table>
<thead>
<tr>
<th></th>
<th>Fe (ng/mL)</th>
<th>Mn (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (1×)</td>
<td>2.3</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>0.1% Pluronic + PBS</td>
<td>2.5</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>PBS + 2 mM EDTA + 0.5% BSA</td>
<td>8</td>
<td>&lt; 0.5</td>
</tr>
</tbody>
</table>

Table 7.4 Fe and Mn content in suspending media analyzed by ICP-MS
Figure 7.1 HeLa cell growth curve (blue diamonds), viability curve (red triangles), and MM curve (black circles). Error bar represents one standard deviation.

The time course for a selected cell line is shown in Figure 7.1. It shows MM, viability, and cell concentration results of HeLa cells during eight-day cultivation without media exchange (no cell passaging). The mean MM of HeLa cells is slightly positive, which is different from the slightly negative mean MM of the oxygenated RBC used as reference, Figure 5.5. Note drop in cell viability after day 3 in culture. Note cell growth inhibition after day 4 in culture. These results suggest that the inclusion of the increasing number of non-viable cells in the sample does not affect its mean MM (note absence of change in mean MM with time of the experiment). Consequently, these results suggest that the death of the HeLa cell does not affect its magnetophoretic mobility. The mean
MM of HeLa cells was compared with that of another cell line. Figure 7.2 (A) shows an example of the MM distribution of U-937 cell after 24-hour cultivation with viability of 90.9%; note that it is very similar to the oxygenated RBC’s MM histogram shown in Figure 5.5. For comparison, Figure 7.2 (B) is an example of the MM distribution of HeLa cell after 19-hour cultivation with viability of 96.1%. For almost the same incubation time, more HeLa cells with the positive MM were observed as compared with the corresponding number of the U-937 cells.

The skewed distribution of the MM data of these K-562 and HeLa cells suggests the presence of high mobility fraction in the population of low MM cells. By exploring the MM data carefully, we found that the significant magnetophoresis was caused by about 1-2% of cells in HeLa and K-562 cell suspensions. In order to further characterize the high mobility cell fraction, we have defined it as more magnetophoretically mobile than our reference “non-magnetic” cells, the oxygenated erythrocytes. The cut-off MM separating “non-magnetic” from “magnetic” cell fractions, was defined as the 95th percentile MM of oxygenated RBC (see Figure 5.5 in Chapter 5), with the corresponding cut-off MM value $= 2 \times 10^{-5}$ mm$^3$/TAs. Consequently, the re-analysis of data in Figure 7.2 shows that about 2% cells are magnetic in HeLa cells shown in Figure 7.2 (B), compared with 0% magnetic cell fraction in U-937 cells shown in Figure 7.2 (A).
Figure 7.2  (A) MM histograms of U-937 cells after 24-hour incubation, and (B) HeLa
cells after 19-hour incubation in complete media.
Figure 7.3  Box plots of MMs of all the eight cancer cell lines incubated in the complete media, and oxygenated RBC. The number is the sample size of each group; the boundaries of the box closest to zero and farthest from zero indicate the 25th and 75th percentiles; whiskers (error bars) above and below the box indicate the 90th and 10th percentiles; a line within the box marks the median; circle symbols are the 5th and 95th percentiles.

The MMs of all the eight cancer cell lines are shown in Figures 7.3 and 7.4 and compared with that of the oxygenated RBC. For every cell line, only samples with the viability greater than 50% were statistically analyzed. For large samples (the sample size of over 1,000) the normality test becomes irrelevant, because a normality test may detect statistically significant but experimentally unimportant deviations from normality. Therefore, both non-parametric and parametric methods were used. Non-parametric method, Kruskal-Wallis test compared the MM medians among cell lines. The following
observations were made. MM median of HeLa cells was significantly greater than all other cells. Both HL 60 and Hep G2 had the smallest MM medians. Student’s t-test was also carried out and results are shown in Figure 7.4. HeLa and K-562 cells exhibit significant higher magnetophoresis compared to all other cell lines and the mean MM of HL 60 is the smallest. The broad and asymmetric MM distribution of K-562 cell makes its median MM significantly greater in t-test, but not in non-parametric test. Overall, the two statistical tests suggest that a portion of HeLa and K-562 cells may possess high intrinsic magnetic susceptibility, as discussed above.

Figure 7.4 Comparison of mean MMs between all the eight cancer cell lines incubated in the complete media, and oxygenated RBC. Red circles highlight the most significant ones.
7.3.4 MM of cells incubated in the complete media with 0.5 mg/mL Fe(NO$_3$)$_3$ addition

In order to test the effect of the elevated soluble iron in the media, we have added soluble iron compounds to the media based on the literature (Hirsh et al. 2002; Neumannova et al. 1995; Popovic and Templeton 2004). Two different soluble iron compounds typically used for studies on iron transfer in cells were used, ferric nitrate, Fe(NO$_3$)$_3$, and ferric ammonium citrate (FAC, described in the next section). Fe(NO$_3$)$_3$ with a final concentration of 0.5 mg/mL (~2.1 mM) was added to the complete media of the selected cancer cell suspensions. After inoculation, cell samples were taken every 12 to 24 hours for cell counting, viability determination, and MM measurement. Before CTV analysis, cells were washed once with PBS containing 0.1% Pluronic F-68, as described above. Figure 7.5 and 7.6 presents the MMs of the six cancer cell lines with the negative and positive controls (oxygenated and metHb containing RBC, respectively). For every cell line, only samples with viability greater than 50% were statistically analyzed, for consistency with the results obtained for cells without iron addition (described above). Again, HeLa cells have shown a significant increase in mean MM after Fe(NO$_3$)$_3$ addition. This is further confirmed by Figure 7.7, where the mean MM of viable cells in media containing Fe(NO$_3$)$_3$ is about $5 \times 10^{-5}$ mm$^3$/TAs as compared to the mean MM of HeLa cells in the complete media without Fe(NO$_3$)$_3$ addition, $3.5 \times 10^{-6}$ mm$^3$/TAs. Interestingly, here the mean MM decreases when the viability of the HeLa decreases, unlike the results obtained without the iron addition, described above. Both Kruskal-Wallis test and t-test show that the MM of HeLa cells is significantly higher than all other cells.
Beside HeLa cells, Hep 3B cells also exhibited a relatively high MM compared to other cell lines. CAL-27 and D562 cells did not grow well in this modified media. Their viability dropped to less than 50% following 20 hours after inoculation. But Fe(NO₃)₃ addition did not change other cells’ growth rate, viability, and cell size.

Figure 7.5  Box plots of MMs of six cancer cell lines incubated in media with Fe(NO₃)₃ addition, oxygenated, and metHb containing RBC. The number is the sample size of each group; the boundaries of the box closest to zero and farthest from zero indicate the 25th and 75th percentiles; whiskers (error bars) above and below the box indicate the 90th and 10th percentiles; a line within the box marks the median; circle symbols are the 5th and 95th percentiles.
Figure 7.6  Comparison of mean MMs between six cancer cell lines incubated in media with Fe(NO₃)₃ addition, oxygenated, and metHb containing RBC. Red circle highlights the most significant one.
Figure 7.7  Comparison of MM (circles) and viability (triangles) of HeLa cells incubated in complete media (dark gray) and media with 0.5 mg/mL Fe(NO$_3$)$_3$ addition (black). Error bar represents one standard deviation.

7.3.5 MM of cells incubated in the complete media with 0.5 mM FAC addition

Another soluble iron compound used for studies on intracellular iron is ferric ammonium citrate (FAC) (Hirsh et al. 2002; Neumannova et al. 1995). FAC was added to media with a final concentration of 0.5 mM (0.132 mg/mL) in the complete media of the tested cancer cell lines in suspension. The same protocol as that used for experiments with ferric nitrate addition was used. After inoculation, cell samples were taken every 12 to 24 hours for cell counting, viability determination, and MM measurement. Before CTV analysis, cells were washed once with PBS containing 0.1% Pluronic F-68. For every cell line, only samples with viability greater than 50% were statistically analyzed.
MMs of the eight cancer cell lines with oxygenated and metHb containing RBCs serving as the negative and positive controls, respectively, are illustrated in Figure 7.8 and 7.9. In contrast to Fe(NO$_3$)$_3$, MM of most cells increased after addition of FAC. This perhaps could be explained by the fact that FAC has a pathophysiological relevance as an Fe donor, and has been shown by others to participate in Fe transfer to physiologically relevant compartments, including ferritin (Richardson and Ponka 1997). MM of the Hep 3B cell was affected the most by FAC addition, and it shows significantly the highest median and mean MM among all the cancer cell lines, as confirmed by Kruskal-Wallis and Student t tests.

![Graph showing comparison of median MMs between eight cancer cell lines incubated in media with FAC addition, and oxygenated and metHb containing RBC. The number is the sample size of each group.](image)

Figure 7.8  Comparison of median MMs between eight cancer cell lines incubated in media with FAC addition, and oxygenated and metHb containing RBC. The number is the sample size of each group.
Figure 7.9  Comparison of mean MMs between eight cancer cell lines incubated in media with FAC addition, oxygenated, and metHb containing RBC. Red circle highlights the most significant one.

7.3.6 TfR1 (CD71) expression

As mentioned in Introduction section, many types of cancer cells with a high rate of proliferation are likely to express more TfR1s than normal cells as they have a higher iron requirement. Therefore, the TfR1 expression level was measured for cells that showed the highest magnetophoretic mobility in the preceding studies, as the high mobility is likely to be related to the increased iron transfer into the cell. The effect of exogenous iron in the form of FAC or Fe(NO₃)₃ on the levels of surface TfR1 in both K-562 and HeLa cells was determined by the binding of PE-conjugated monoclonal anti-human TfR1. The surface TfR1 was quantified by QuantiBRITE™ PE beads, and then normalized to the TfR1 number expressed on control cells (0 hr) which were incubated in
the complete media without iron addition, Figure 7.10. Rather unexpectedly, the treatment of cells with 0.5 mM FAC or 0.5 mg/mL Fe(NO$_3$)$_3$ reduced the number of TfR1s by 25 - 40% within 1-5 hrs incubation time for both K-562 and HeLa cells. The likely explanation is that during this time period, cells were in the lag phase of growth, when they were adapting to the new media. During the adaptation process, some of the surface TfR1 could have become inactive and therefore could not have been detected by the PE antibody. Moreover, HeLa cell, as an adherent cell has a longer lag period than K-562, a suspension culture. Consequently, it took only about 1 hr incubation for the surface TfR1 signal in K-562 cell to rise but a longer time of about 5 hrs for HeLa cell to return to the control TfR1 signal level.

After about 24 hrs incubation, surface TfR1s expression reached the level of control cells for HeLa cells or increased above the control level for K-562 cells. The cells were probably proliferating at this period, as proliferating cells require more irons for their growth and metabolism than resting cells (Koorts and Viljoen 2007). After about 48 hrs, the level of surface TfR1 tended to decrease again. This is likely because TfR1 expression is primarily regulated at the post-transcriptional level in response to the intracellular iron contents. The likely scenario, based on my literature review (Richardson et al. 2009), is that for high intracellular iron conditions, iron regulatory proteins 1 and 2 (IRP1 and IRP2) do not bind to the five iron response elements (IREs) in the 3’ untranslated region (UTR) of the TfR1 mRNA, which leads to TfR1 mRNA degradation, Figure 7.11 (A). Meanwhile, high intracellular iron concentration impedes the binding of IRP1 and IRP2 to the one IRE in the 5’ UTR of mRNA of the iron storage protein ferritin allowing its translation, Figure 7.11 (B). Based on this information, the interpretation of
my experimental data is that the iron uptake by the cell from the media was inhibited by
down-regulation of the TfR1 expression and therefore it promoted the intracellular iron
storage, leading to the increased magnetic moment of the cell and the observed, increased
cell magnetophoretic mobility.

Figure 7.10  Surface TfR1 expression on K-562 (circles) and HeLa (triangles) cells
incubated in media with FAC (solid symbols) or Fe(NO$_3$)$_3$ (open symbols) addition. Data
were presented here relative to the TfR1 expression on control K-562 or HeLa cells
which were incubated in the complete media without iron addition.
Interestingly, the data show that K-562 cells seemed to express more TfR1s than HeLa cells, but the mean MM of K-562 was lower than that of HeLa cells, as described in sections 7.3.3 and 7.3.4. The possible explanation is that the uptake of iron probably does not correlate with the number of TfR1 molecules on the cell surface but rather correlates with the activity of internalization of TfR1 into the cells. This observation agrees with the report by others (Sakaguchi et al. 2008) that TfR1s in K-562 cells were internalized slowly, whereas those in HeLa cells were internalized quickly and actively.

**7.3.7 Enrichment and analysis of magnetic K-562 and HeLa cells**

In order to further explore the mechanism of existence of the 1-2% magnetic cell subpopulation in HeLa and K-562 cells, purification of the magnetic cell fraction is highly desirable. Inspired by the sperm cell separation and magnetophoresis study,
described in Chapter 6, MACS® MS column was used to enrich these rare magnetic cells. Table 7.5 and Figure 7.12 shows one of the MACS separation results on HeLa cells which were cultured in the complete medium (as described above). About 1.1% cells were isolated from cultured HeLa cells with a mean MM of $5.51 \times 10^{-5}$ mm$^3$/TAs and the intracellular Fe content of 428 fg/cell. These values are significantly higher than those of the metHb containing RBC used as a positive control (the mean MM of $4.74 \times 10^{-6}$ mm$^3$/TAs and Fe content of 100.44 fg/cell, correspondingly). The mean MM value and Fe content in the positive fraction were much higher than those in the feed (before separation) and the negative fraction, as expected. The cells recovered in the negative fraction possessed the lowest Fe content and mean MM of all three fractions, again as expected. The cell viability did not change significantly before and after separation. Taken together, these results show the existence of a cell subpopulation with high intracellular iron content that have relatively high intrinsic magnetic susceptibility, which induced them to be retained in the column and resulted in their high mean MM value. But it is still unclear why only a small portion of cells in one culture accumulated so much intracellular iron. The mechanism is probably different from that observed in apoptotic spermatozoa (discussed in Chapter 6). These findings point to potential, new avenues of research on iron metabolism in cancer cells, and suggest new applications of CTV technology to single cell analysis.
<table>
<thead>
<tr>
<th></th>
<th>Cell # (×10^6)</th>
<th>Viability (%)</th>
<th>Mean m (mm^3/TAs)</th>
<th>Fe (fg/cell)</th>
<th>Mn (fg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Before separation</td>
<td>42.4</td>
<td>91.9</td>
<td>3.33×10^{-6}</td>
<td>-</td>
<td>45.65</td>
</tr>
<tr>
<td>Negative fraction</td>
<td>36.7</td>
<td>91.7</td>
<td>-6.92×10^{-7}</td>
<td>-1.56×10^{-8}</td>
<td>38.46</td>
</tr>
<tr>
<td>Positive fraction</td>
<td>0.46</td>
<td>81.7</td>
<td>5.51×10^{-5}</td>
<td>1.38×10^{-5}</td>
<td>428.02</td>
</tr>
<tr>
<td>metHb RBC</td>
<td>-</td>
<td>-</td>
<td>4.74×10^{-6}</td>
<td>100.44</td>
<td>&lt; 0.04</td>
</tr>
</tbody>
</table>

A, data was obtained directly after separation. B, the isolated cells were recultured for about 72 hrs and then analyzed by CTV and ICP-MS

Table 7.5 MACS separation results on HeLa cell

![Figure 7.12 MM histograms of HeLa cell before and after separation.](image)

Continued
In a follow-on study, the isolated positive and negative cells were recultivated in the same complete media as the original cell line. After about 72 hrs incubation, cells were harvested and analyzed by CTV and ICP-MS again. Rather unexpectedly, the isolated positive cells appeared to lose their magnetic attributes during incubation, which
was reflected in the reduced value of MM and a lower average Fe content. Mean MM was reduced from $5.51 \times 10^{-5}$ to $1.38 \times 10^{-5}$ mm$^3$/TAs, and Fe content decreased from 428 to 159 fg/cell. No significant changes were found in the negative cell fraction following recultivation in the same conditions. Again, the mechanism of the associated changes in the cellular metabolism is not known at this time, but they present an interesting opportunity for a new line of research in future studies.

<table>
<thead>
<tr>
<th>Cell # (×10$^6$)</th>
<th>Viability (%)</th>
<th>Mean m (mm$^3$/TAs)</th>
<th>Fe (fg/cell)</th>
<th>Mn (fg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
<tr>
<td>Before separation</td>
<td>26.6</td>
<td>91.2</td>
<td>3.37×10$^{-6}$</td>
<td>-</td>
</tr>
<tr>
<td>Negative fraction</td>
<td>26.5</td>
<td>89.4</td>
<td>-1.26×10$^{-6}$</td>
<td>-7.78×10$^{-8}$</td>
</tr>
<tr>
<td>Positive fraction</td>
<td>0.26</td>
<td>75.3</td>
<td>5.36×10$^{-5}$</td>
<td>1.60×10$^{-5}$</td>
</tr>
</tbody>
</table>

A, data was obtained directly after separation. B, the isolated cells were recultured for about 72 hrs and then analyzed by CTV and ICP-MS

Table 7.6 MACS separation results on K-562 cell

The significance of these new findings was further strengthened by similar separation results obtained for cultured K-562 cells, Table 7.6. Before separation, cells possessed 47.54 fg Fe/cell on average. After separation, the negative cells contained
39.96 fg Fe/cell on average and the positive cell fraction contained 326.96 fg Fe/cell on average. Fractional cell viability did not change before and after separation either. Similarly as observed for the HeLa cells, the mean MM and Fe content in the positive cell fraction decreased after reculture.

<table>
<thead>
<tr>
<th></th>
<th>Cell # ($\times10^6$)</th>
<th>Viability (%)</th>
<th>Mean $m$ (mm$^3$/TAs)</th>
<th>Fe (fg/cell)</th>
<th>Mn (fg/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before separation</td>
<td>49.6</td>
<td>85.9</td>
<td>-2.49$\times10^{-7}$</td>
<td>17.12</td>
<td>0.82</td>
</tr>
<tr>
<td>Negative fraction</td>
<td>45.4</td>
<td>89.3</td>
<td>-1.01$\times10^{-7}$</td>
<td>13.47</td>
<td>0.66</td>
</tr>
<tr>
<td>Positive fraction</td>
<td>-*</td>
<td>10.3</td>
<td>3.11$\times10^{-5}$</td>
<td>~751.76*</td>
<td>~8.68*</td>
</tr>
</tbody>
</table>

*almost all cells were dead, which made the visual counting difficult. The Fe and Mn concentrations were just calculated by an estimation of the cell number.

Table 7.7 MACS separation results on HL-60 cell

For comparison, MACS separation was also performed on HL-60 cells that consistently showed low mean MM, independent of Fe addition to culture media (Figures 7.3 and 7.4) and lacked any evidence of the presence of the “magnetic” cell subpopulation. In contrast to HeLa and K-562 cells after separation, almost all HL-60 cells captured by the column were dead with a fractional viability of only about 10%. Consistent with the function of the magnetic separation column, the positive cells had higher Fe content and mean MM value than the feed (before separation) and the negative cells. This phenomenon of the increased mean MM value and the intracellular Fe content
in apoptotic (non-viable) cells is very similar as that found in spermatozoa separation discussed in Chapter 6. There, the apoptotic or dead cells were retained in the MACS column as a result of elevated iron or ROS content.

Compared to the intracellular Fe content, much lower Mn content was observed in each sample, Tables 7.5-7.7. If for instance all irons (45.65 fg/cell) in HeLa cells are in the form of FeO with a magnetic susceptibility of $7,180 \times 10^{-6}$ (Weast 1981), and all manganese (1.74 fg/cell) in HeLa cells are in the form of MnO with a magnetic susceptibility of $4,680 \times 10^{-6}$ (Weast 1981), the contribution of FeO and MnO to the bulk cell magnetic susceptibility are $3.58 \times 10^{-8}$ and $9.34 \times 10^{-10}$ respectively. Hence, the contribution of Mn to the cell magnetophoresis was neglected.

### 7.3.8 Cell cycle analysis

Iron plays an important role in the DNA synthesis. Cells at one specific phase may uptake more iron than those at other phases. A possible link between the cell cycle phase and the fractional concentration of the high MM cell fraction was examined by analyzing cellular DNA content in HeLa cells before and after MACS separation. The cellular DNA content was measured by fluorescent stain binding. As propidium iodide (PI) binds stoichiometrically to the double-stranded DNA, the fluorescent intensity of a stained cell offers a direct measure of the DNA content in that cell. Cells in G0 or G1 phases possess a normal diploid DNA content, which are represented by the narrow peak on the left side of the DNA histogram, Figure 7.13. Cells in G2 or M phases contain twice the normal DNA content, and are represented by the second, smaller peak located on the right side of the distribution with twice the cell fluorescence intensity value. As the
DNA is synthesized during S phase, the resulting cell fluorescence intensity is distributed continuously between G0/G1 and G2/M phases in Figure 7.13. Table 7.8 shows the percentage of cells in each phase of the cell cycle. No difference in the cell cycle distribution was found between feed (before separation) and the magnetically fractionated HeLa cells.

![Figure 7.13 Cell cycle analysis on HeLa feed by program ModFit™](image)

<table>
<thead>
<tr>
<th></th>
<th>G0/G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before separation</td>
<td>85.56</td>
<td>13.63</td>
<td>3.80</td>
</tr>
<tr>
<td>Negative fraction</td>
<td>83.06</td>
<td>13.50</td>
<td>3.44</td>
</tr>
<tr>
<td>Positive fraction</td>
<td>80.69</td>
<td>14.71</td>
<td>4.60</td>
</tr>
</tbody>
</table>

Table 7.8 Percentage of cells in each phase of the cell cycle for HeLa cells before and after separation.
7.4 Conclusions

Iron metabolism study in cancer cells is of particularly interest because of the potential diagnostic applications and new anti-cancer therapeutics development. In this study, eight cancer cell lines were screened in search of the naturally-occurring, magnetic field-induced cell motion that could be related to abnormal iron metabolism in certain cancers. The results showed that small fractions of HeLa and K-562 cells are magnetic when they were cultured in the complete cell culture media without extra iron compound addition. The magnetic cell fraction was successfully enriched by a label-less magnetic separation, and showed to possess higher MM and iron content than the unenriched cell fraction. Interestingly, their magnetic property was lost after cultivation. No difference in cell cycle distribution was found between these magnetic cells and the control (unseparated) cells. Reasons why there exists a small portion of magnetic HeLa and K-562 cells containing high intracellular iron present an interesting topic for further studies.

It was also observed that extra iron compound addition to cell culture media can significantly increase MM of some cell lines without altering their growth rate, viability, and cell size. In contrast, it caused some other cell lines to die. The effect of different iron compounds was different. This is likely caused by different mechanisms of iron uptake by cancer cells as described in the published literature. Difference in the magnetic susceptibility between different cancer cells as a result of exposure to different iron compounds in the culture media establishes the possibility of their separation without targeting by any immunomagnetic labeling reagents but rather by targeting selected metabolic pathways.
The changes in the intracellular iron content as measured by single cell CTV analysis and corroborated by the bulk cellular Fe content analysis by ICP were consistent with the known molecular mechanism of iron transfer into the cell. During 1-5 hrs incubation, the cell adaptation process to new culture media conditions after iron compound addition caused a downregulation or inactivation of surface TfR1 levels in both HeLa and K-562 cells. After that, the cells have shown evidence of an increased iron uptake with a concomitant increase in surface TfR1 expression. Later on, the high intracellular iron condition induced downregulation of the TfR1 levels again in both cell lines. The data also agree with other reports that TfR1s in HeLa cells were internalized more quickly and actively than those in K-562 cells.

Thus, it has to be emphasized here that cells with high MMs detected by CTV are very likely caused by the Fe internalization and accumulation rather than iron cell surface adhesion.
CONCLUSIONS AND RECOMMENDATIONS

8.1 Conclusions

The present study was focused on the detection of cell motion in suspension in imposed magnetic fields and gradients, unaided by attachment of immunomagnetic tags. This work is an outgrowth of the currently used magnetic cell analysis and separation techniques relying on selective cell magnetization by attachment of synthetic magnetic micro- or nano-particles. Magnetophoretic mobility (MM) was utilized as a measure of this type of cell motion, or cell magnetophoresis, irrespective of whether it is native or imparted, that can be quantitatively determined by CTV. Evaluation of MM has been employed to select immunomagnetic labeling reagents having high magnetization capacity for immunomagnetic sorting (Jing et al. 2007; Yang et al. 2009). It has also been successfully used for quantitative determination of the specific cell surface markers, cell surface and intracellular magnetic nanoparticle tagging (Jing et al. 2008; McCloskey et al. 2000; McCloskey et al. 2001a; McCloskey et al. 2001b). In these past studies, cell MM was very significant due to this magnetic tagging.
Most cells in nature are diamagnetic due to diamagnetic properties of their major components (mostly water) and exhibit very weak intrinsic cell magnetophoresis. In order to detect such weak phenomena of naturally occurring cell motion, MM theory and CTV instrumentation and protocols were further perfected. The required high accuracy of the CTV instrument was first tested and confirmed by examining the Browninan motion of monodisperse microspheres. The CTV instrument was further revised to allow the measurement of cell/particle settling velocity (or sedimentation coefficient) simultaneously with magnetically induced velocity (or MM). Settling velocity is directly associated with cell/particle size. This improvement provides a more flexible way to differentiate cell subsets, based on not only cell magnetophoresis, but also cell size.

Moreover, the improved CTV system was demonstrated to be sensitive to the type of the microparticle magnetization, and is capable of distinguishing between the motion of magnetically unsaturated species (paramagnetic or diamagnetic) and magnetically saturated species (superparamagnetic). MM models for different materials were established. The paramagnetic and diamagnetic magnetophoresis model has been verified experimentally using synthetic microparticles and Bacillus globigii spores and the CTV analysis. The model predicted distinctly different behavior for the superparamagnetic-labeled microparticles, and this was also confirmed experimentally. The understanding of the underlying, physical mechanisms of these two different motions is important for applications to magnetic cell separation and the detection of magnetic species in the cell in health and disease.

A fluorescent CTV system (Fl CTV) was also developed to detect the cell fluorescent signals conferred by the tagged primary antibodies. In practice, due to
reaction kinetics and the reagent chemistries, one observes four subpopulations \((F^+M^-, F^+M^+, FM^-, FM^+)\) following a two-step labeling process. The combination of the regular VIS CTV and Fl CTV provides a unique opportunity to investigate the size of each fraction and sensitivity and specificity of commercially available or newly synthesized magnetic colloids. So far, the potency of our CTV system is being greatly increased by detecting three parameters: MM, sedimentation coefficient, and fluorescence, which is comparable to flow cytometry. CTV, however, requires lower capital and operating costs.

In the second part of this study, intrinsic cell magnetophoresis and label-less magnetic separation were investigated. It was shown theoretically that it is possible to detect and measure the magnetic field-induced motion of the RBCs because of their hemoglobin (Hb) iron content. The unpaired electron spins in the four heme groups of deoxyHb and metHb contribute to a paramagnetic moment in deoxy and metHb-containing RBCs. Oxy RBCs do not have unpaired electrons, and are more diamagnetic compared to suspending aqueous media. Differences in MM between metHb RBCs and oxy RBCs were predicted and confirmed by CTV analysis. The paramagnetic behavior of deoxy RBCs relative to the suspending media has been exploited to isolate the cultured RBCs from hematopoietic stem cell (HSC) bioreactor cultures which are diamagnetic. CTV analysis also provides a new way to quantify the intracellular Hb concentration in each cultured or donor-derived RBC. By combining the magnetophoresis and size analysis by CTV, morphology analysis by differential interference contrast (DIC) microscopy, and deformability analysis by ektacytometry, we found that batch separation on a MACS system could separate cultured RBCs with high purity, but very low
throughput. The results also indicate that the isolated positive cells contain similar amount of Hb as donor-derived RBC, but have larger size.

The label-less magnetic separation method for selection of non-apoptotic human spermatozoa for potential, future application to assisted fertilization was proposed and studied. It is suggested that apoptotic spermatozoa probably have a higher magnetic susceptibility than those non-apoptotic, motile cells, caused by the elevated intracellular iron and or ROS content. This new method provides a comparable separation performance to the immunomagnetically labeled method, and avoids the possible contamination of magnetic beads to the selected population.

The described label-less separation studies led to the investigation of the naturally-occurring magnetophoresis of cancer cells. The results revealed the existence of small subpopulations (1-2%) in HeLa and K-562 cell lines with high intrinsic magnetic susceptibility, further confirmed by their isolation (without labeling) using a MACS system. Moreover, the effect of iron compound introduction to cell culture media on the intracellular iron transport was found to be different for different cancer cell lines. Difference in the magnetic susceptibility between different cancer cells as a result of exposure to different iron compounds in the culture media establishes the possibility of their separation without targeting by immunomagnetic labeling reagents.

8.2 Recommendations

Future work should focus on further improvement of the CTV system, study of the mechanism of observed intrinsic magnetophoresis phenomena in apoptotic spermatozoa and some cancer cells, and investigation of separation scale-up.
8.2.1 Development of higher gradient CTV

A CTV system with higher magnetic field and field gradient (higher than the current design where $B_0 = 1.260 \pm 0.115$ T, $\text{grad } B_0 = 0.140 \pm 0.012$ T/mm, and $\text{grad } (B_0^2) = 0.35$ T$^2$/mm at the viewing area) should be developed to improve its sensitivity for weak intrinsic magnetophoresis measurement. Applying the high energy permanent magnet materials (NdFeB), one could design and assemble a higher field and gradient CTV system with the aid of computer modeling field software. This may also be achieved by an array of permanent magnets or magnetic dipoles which are arranged to provide a high strength, high gradient magnetic field adjacent the surface of the array.

8.2.2 ROS measurement

ROS and oxidative stress can induce carcinogenesis and cell apoptosis. It has been suggested that elevated intracellular ROS content may contribute to the high intrinsic magnetic susceptibility of apoptotic spermatozoa and the identified HeLa and K-562 cells. This hypothesis should be examined by quantitatively measuring various ROS. However, most ROS are highly reactive and short lived. It is a significant analytical challenge to measure ROS in cells and living organisms directly. A method of utilizing molecular probes and flow cytometry is suggested. Molecular probes offer derivatives of reduced fluorescein and calcein as cell-permeant indicators for ROS. This method is sensitive but limited by the short-lived ROS. An alternative method involving combination of the spin trapping technique to produce more stable radicals with electron spin resonance (ESR, sometimes called electron paramagnetic resonance, EPR) is also recommended. ESR is a spectroscopic technique that detects unpaired electrons and is
specific for free radicals. Very few quantitative results have been reported for ESR, and simultaneous assessment of various ROS using the both methods may be advisable.

### 8.2.3 Fe and Mn oxidation state study

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\chi \times 10^6$</th>
<th>Temperature (K)</th>
<th>Molecular weight</th>
<th>Density (g/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FeO</td>
<td>7,180</td>
<td>293</td>
<td>71.85</td>
<td>5.7</td>
</tr>
<tr>
<td>Fe$_2$O$_3$</td>
<td>1,480</td>
<td>1033</td>
<td>159.69</td>
<td>5.24</td>
</tr>
<tr>
<td>Fe$_3$O$_4$</td>
<td>1,790,000</td>
<td>293</td>
<td>231.54</td>
<td>5.1</td>
</tr>
<tr>
<td>$\alpha$ Mn</td>
<td>870</td>
<td>293</td>
<td>54.938</td>
<td>7.2</td>
</tr>
<tr>
<td>$\beta$ Mn</td>
<td>795</td>
<td>293</td>
<td>54.938</td>
<td>7.2</td>
</tr>
<tr>
<td>MnO</td>
<td>4,680</td>
<td>293</td>
<td>70.94</td>
<td>5.43-5.46</td>
</tr>
<tr>
<td>Mn$_2$O$_3$</td>
<td>5,051</td>
<td>293</td>
<td>157.87</td>
<td>4.5</td>
</tr>
<tr>
<td>MnO$_2$</td>
<td>1,656</td>
<td>293</td>
<td>86.94</td>
<td>5.026</td>
</tr>
<tr>
<td>Mn$_3$O$_4$</td>
<td>3,307</td>
<td>298</td>
<td>228.81</td>
<td>4.856</td>
</tr>
</tbody>
</table>

Volume magnetic susceptibilities in SI units were calculated from molar magnetic susceptibilities in CGS units provided by the CRC Handbook of Chemistry and Physics (Weast 1981) with the following equation: $\chi (\text{SI}) = 4\pi \rho \chi_m (\text{CGS})/M_w$.

Table 8.1 Volume magnetic susceptibilities in SI units

Besides quantifying the amount of Fe and Mn in cells by ICP-MS, the oxidation state of the Fe and Mn should be analyzed. Significant ranges of magnetic susceptibility of the various forms of Fe and Mn are listed in Table 8.1. Both Fe and Mn have significant magnetic properties, which are strong functions of the oxidation state of the
metal atom. Various oxidation states of Fe and Mn contribute differently to the cell magnetic susceptibility. X-ray photoelectron spectroscopy (XPS), a quantitative spectroscopic technique, or Mössbauer spectroscopy, could be used to measure the elemental composition and chemical state of the elements within a material. Then, based on the information about the fractional concentration of different oxidation states of the intracellular Fe and Mn, one could calculate the paramagnetic metal contribution to the cell volume magnetic susceptibility and MM theoretically, which could be compared with the measured cell MM data.

8.2.4 Determination of iron-binding proteins in “magnetic” cancer cells

The expression of iron-binding proteins such as ferritin and transferrin receptor 1 in “magnetic” K-562 and HeLa cells should be determined and compared to non-magnetic cancer cells. Higher iron-binding protein expression in “magnetic” cancer cells is expected. This could be carried out by quantifying protein mRNA levels through real-time polymerase chain reaction (qRT-PCR). qRT-PCR uses fluorescent reporter dyes to monitor the amplification products during each cycle of the PCR, and combines the DNA amplification and detection steps into one assay (Higuchi et al. 1993). It has the ability to detect the presence and quantity of specific nucleic acid sequences. K-562 and HeLa cells cultivated in the complete media are first subjected to magnetic cell separation. RNAs of the enriched “magnetic” and non-magnetic cells are isolated followed by reverse transcription to make cDNA with the aid of synthesized target-specific primers. The number of cDNA transcripts present for a specific gene is then determined.
8.2.5 Scale-up of the separation process

A fast separation process with high throughput is necessary for the cultured RBC and “magnetic” cancer cell separation. Increasing the uploaded cell concentration has led to clogging of MACS columns and reduction of separation efficiency. Continuous sorting systems such as QMS, described in Chapter 1, should be used in preference to the batch separation systems. Due to the weak native magnetophoresis of RBCs and cancer cells, a higher magnetic gradient is indicated as a requirement for QMS. The higher gradient increases the MM difference between the target cells and unwanted cells, which will result in increased recovery and purity of the target cell. Meanwhile, a higher field gradient increases the positive cell velocity, and therefore allows a higher total flow rate to be used in the separation. For post differentiation separation of cultured RBC, the magnetic separation should be integrated with deoxygenation, which will greatly shorten the process time and reduce the labor intensity. Additionally, multiple QMS systems in series should be considered as an option to increase the throughput of RBC separation.
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APPENDIX A

HARDWARE AND SOFTWARE COMPONENTS OF THE VARIABLE-FIELD CTV SYSTEM

A.1 Calibration and zeroing of electromagnet

The high magnetic fields generated in the CTV system (on the order of 1 tesla) results in a residual magnetic field in the steel pole pieces of the CTV instrument. This is not a problem when permanent magnets are used, but results in significant uncertainty in the actual field strength when the electromagnet is repeatedly turned on and off. Consequently, an electric current switching system was incorporated which results in a reversal of the current, and thereby the polarity, of the applied magnetic field, leading to the desired steel pole piece demagnetization. Since the intent of the addition of the electromagnet was to be able to accurately vary the magnetic energy density gradient in an automatic manner, a relationship of current to magnetic field, and correspondingly, the magnetic energy density gradient, was needed. This spatial field calibration was developed using a Hall effect probe attached to the Gauss meter mounted on a stage of a programmable syringe pump (Model 71-2102, Harvard PHD 2000, Hollison, MA). A computer controlled auto-calibration program was also developed and used to measure
the magnetic field along the x-axis and y = 0 plane of symmetry (Figure 3.2A). The probe was initially aligned with the top edge of the pole piece (x = 0) and measurements were recorded at intervals of 0.25 mm from 4 mm to 14 mm. The magnetic flux density, $B_0$, was measured as a function of the electric current in the coils of the electromagnet and recorded directly into the host computer. At each x position, measurements were made at currents ranging from -0.08 A to 5.0 A at intervals of 0.2 A through each coil.

### A.2 Calibration of CTV electromagnet

Figure A.1 (A) shows plots of the experimentally measured magnetic induction, $B_0$, as a function of the Gauss meter probe position on the magnet’s plane of symmetry (y = 0, Figure 3.2 (A)) along x axis, for different settings of the electric current powering the electromagnet, ranging from -0.08 to 5.0 A. These experimental measurements, squared, were then fitted to a cubic polynomial curve at each current, Figure A.1 (B). Calculations of $\frac{dB_0^2}{dx}$ as a function of position spanning the CTV region of interest, ROI (x = 4 to 14 mm, y = 0) and the current powering the electromagnet, were made as shown in Figure A.2. The ROI of the CTV was centered at 8.5 mm, the flattest region of the curves, and therefore a region of the nearly constant $S_m$ that best approximates the isodynamic condition of the magnetic field. At $x = 8.5$ mm, a sixth-order polynomial was used to fit $\frac{dB_0^2}{dx}$ data to the current. From the expression for $S_m$ in eqn (3.2), the dependence of $S_m$ on the electric current powering the electromagnets, at the center of the ROI, is then calculated and presented in Figure 3.3.
Figure A.1 Experimentally measured $B_0$ field (A) and calculated $B_0^2$ (B) on the plane of symmetry $y = 0$, along $x$ axis, at 27 different values of the electric current powering the electromagnet coils ($I = -0.08, 0.00, 0.20, 0.40, \ldots, 4.80, 5.00$ A).
Figure A.2 Values of $dB_0^2/dx$ on the plane of symmetry $y = 0$ over a range of $x$ covering the CTV region of interest (ROI), as a function of the electric current in the coils. (Note 27 different current values tried, from -0.08 A, 0.00 A to 5.00 A at intervals of 0.20 A.)

### A.3 CTV software

Significant improvements in the interface of the CTV software have also been implemented. The CTV software consists of two main parts: an image acquisition part and an image processing and data analysis part. An overall outline of how the host computer controls the hardware involved is shown in Figure A.3. With GPIB and RS-232 programmable interface cards, the CTV software allows the user to set up the desired magnetic energy density gradient, $S_m$, based on previously determined calibration curves of magnetic energy density gradient and current. In addition, the software can also control the Harvard syringe pump allowing automatic delivery of samples into the CTV.
viewing region. A Gauss meter (Model 6010, F.W. Bell, Orlando, Florida) placed near to the testing channel is included to allow the user to monitor or record the magnetic induction, $B_0$, during experiments.

Figure A.3 Diagram depicting the flow of information from CTV host computer to various hardware components.

Significant improvements have also been implemented in the actual operation of the CTV program. A flow chart outlining the program algorithm is provided in Figure A.4. The current version, written in C++, runs on a Gateway E-4400 host computer with 512 Mbytes of RAM that can store in excess of 600 frames and an 80 Gbytes hard drives
for image archiving. Consecutive images can be captured by a Cohu CCD camera (Model 4915, San Diego, CA) with the sampling frequency of 30 f/s. The images from the camera were digitized by an M-Vision 1000 PCI 8-bit Video Digitizer (MuTech, Billerica, MA) with resolution of $640 \times 480$ pixels. The magnification of the microscope produces the spatial resolution of the system in the present experiment of $2.7 \, \mu m$ per pixel. The current version allows the user to choose different frame rates under different operating conditions, including field on and off, thereby allowing the magnetically induced velocity, $u_m$, and the settling velocity, $u_s$, to be determined, respectively, for the same particle.

The second part of the CTV software is for image processing and data analysis. It consists of five parts: 1) image pre-processing, 2) cell (or particle) identification, 3) cell tracking, 4) cell velocity calculation, and 5) statistical data analysis. Because of the noisy background and small size of cells, it is almost impossible to distinguish the moving cells from a single image. To overcome this difficulty a dynamic mean-field method is utilized which enables the background to be removed from the series of images. Other commonly used image processing functions are included in this latest version of the CTV code such as erode, dilate, edge, negative, contrast, contour, and threshold. In addition to the raw images, this new version also can read in and convert to different formatted images such as bmp, tiff, jpg, and png. It is also able to make digital format videos (avi) for further studies, presentations, or reports.
Figure A.4  Flow chart for the operation of the CTV program
### APPENDIX B

#### MEDIA AND CELL LINE INFORMATION

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<th>30-2001&lt;sup&gt;a&lt;/sup&gt; RPMI 1640</th>
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<th>30-2003&lt;sup&gt;a&lt;/sup&gt; EMEM</th>
<th>10-022-C&lt;sup&gt;b&lt;/sup&gt; AMEM</th>
<th>14-500&lt;sup&gt;c&lt;/sup&gt; RPMI 1640</th>
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|------------|-----------------|-----------------|----------------|----------------|
| **Vitamins** | | | | |
| Ascorbic Acid | - | - | - | - | - | - |
| D-Biotin | 0.00020 | 0.000013 | - | 0.00010 | 0.00020 | - |
| D-Calcium Pantothenate | - | - | - | 0.00100 | 0.00025 | 0.00400 |
| Choline Chloride | 0.00300 | 0.00400 | 0.00100 | 0.00100 | 0.00300 | 0.00400 |
| Folic Acid | 0.00100 | 0.00400 | 0.00100 | 0.00100 | 0.00100 | 0.00400 |
| I-Inositol | - | - | - | 0.00200 | 0.03500 | 0.00720 |
| myo-Inositol | 0.03500 | 0.00720 | 0.00200 | - | - | - |
| Nicotinamide | 0.00100 | 0.00400 | 0.00100 | 0.00100 | 0.00100 | 0.00400 |
| p-Amino Benzoic Acid | 0.00100 | - | - | 0.00100 | - | - |
| D-Pantothenic Acid | 0.00025 | 0.00400 | 0.00100 | - | - | - |
| (hemicalcium) Pyridoxine·HCl | 0.00100 | 0.00400 | 0.00100 | 0.00100 | 0.00100 | 0.00400 |
| Riboflavin | 0.00020 | 0.00040 | 0.00010 | 0.00010 | 0.00020 | 0.00040 |
| Thiamine·HCl | 0.00100 | 0.00400 | 0.00100 | 0.00100 | 0.00100 | 0.00400 |
| Vitamin B-12 | 0.000005 | 0.000013 | - | 0.000136 | 0.000005 | - |
| **Other** | | | | | |
| D-Glucose | 4.50000 | 4.50000 | 1.00000 | 1.00000 | 2.00000 | 4.50000 |
| Glutathione (reduced) | 0.00100 | - | - | - | 0.00100 | - |
| HEPES | 2.38300 | 5.95800 | - | - | - | - |
| Lipoic Acid | - | - | - | 0.00020 | - | - |
| Phenol Red, Sodium Salt | 0.00500 | 0.01500 | 0.01000 | 0.01000 | 0.00500 | 0.01500 |
| Sodium Pyruvate | 0.11000 | 0.11000 | 0.11000 | 0.11000 | - | 0.11000 |

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<sup>a</sup> from ATCC; <sup>b</sup> from Mediatech; <sup>c</sup> from Central Cell Services, CCF

Table B.1  Media formulation
The ATCC website of each cancer cell line used in this study

U937:
http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.asp x?ATCCNum=CRL-1593.2&Template=cellBiology

K-562
http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.asp x?ATCCNum=CCL-243&Template=cellBiology

HL-60
http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.asp x?ATCCNum=CCL-240&Template=cellBiology

Hep 3B 2.1-7
http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.asp x?ATCCNum=HB-8064&Template=cellBiology

Hep G2
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CAL 27
http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.asp x?ATCCNum=CRL-2095&Template=cellBiology

Detroit 562
http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.asp x?ATCCNum=CCL-138&Template=cellBiology

HeLa
http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.asp x?ATCCNum=CCL-2&Template=cellBiology