Optical Force Based Cancer Cell Identification

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OPTICAL FORCE BASED CANCER CELL IDENTIFICATION

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Justin Michael Roman
December 2007

Accepted by:
Bruce Z Gao, Ph.D., Committee Chair
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We present the basis of a novel, non-invasive technique for cell diagnostics which utilizes the optical force generated by a weakly focused laser beam to distinguish cells based on their size, structure, composition, and membrane properties. Cell populations of different types, biological states or with different treatments can be studied. This research focuses on two particular instances where other methods of cell sorting, such as those that require fluorescent markers, are not ideal. What’s more, this research emphasizes the ability to sort morphologically similar cells that are identical to the naked eye, but phenotypically different on the molecular level. The first study consists of sorting genetically modified cells (Ave. Velocity = 35.5µm/s) from their unmodified phenotype (56.7µm/s). A second study was conducted to show the system’s ability to distinguish cancerous cells from one another. Metastatic cancer (50.1µm/s), non-metastatic cancer (21.4µm/s), and healthy (161.7µm/s) murine breast cells were measured to also be significantly different from one another. Thus, a general live-cell analysis technique allowing detection of small cell-based changes or differences, without additional cell manipulation, was developed.
ACKNOWLEDGMENTS

I would like to thank Dr. Gao for his outstanding guidance, and more importantly, patience, with this project. He has been an amazing advisor and I am sure that maintaining a relationship with him throughout my career will be very beneficial. I would also like to thank my committee members, Dr. Burg and Dr. Wei, for their patience, support, and willingness to provide access to their cell lines. Finally, without my fellow lab members this research would have never progressed to what it has become. I would especially like to thank Kirk Pirlo for his support with the laser, Cassie Gregory for her help with cell culture, Cheryl Parzel for breast cancer cell line support, and finally Hari Kotturi for genetically modified cell support.
DEDICATION

Without the support of my Mom and Dad, my education would have never reached this point in the first place. They have been my inspiration throughout my years in college and I have always done my best to make them proud. They have raised me to always take that extra step and it shows in all the successes I have had so far. My sister, Wendy, who has done everything she can to keep me sane throughout this process has done just that. Although sometimes I think it is her that needs the sanity check. My brother, Matt, whom I am in graduate school because of has showed me what hard work and determination will get you in life. I hope that both my brother and sister realize that I have been simply trying to keep up with them throughout the years. Therefore, this thesis is dedicated to my family and for everything they have done for me over the years. I love you guys.
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CHAPTER ONE

INTRODUCTION

1.1 Background

In the fields of biomedical research[1], diseases diagnoses[2] and treatments[3], the ability to distinguish phenotypically different cells from one another is a particularly useful technique.

There are many biological research topics that require purified samples of cells in order to produce consistent and reliable results[4]. Clinical applications include the screening of a heterogeneous sample of cells for one particular cell type, in many cases a diseased state cell, to either confirm, justify or distinguish a particular disease or condition[5-10].

Cell sorting systems have been designed and applied in many fields of biological research and clinical medicine. For instance, flow cytometry, a device for heterogeneous cell-separation, has had an especially important impact on biomedicine. Although this technique, along with a few others, is very valuable, there are limitations of its application such as the need to use specific cellular markers or using meticulous protein excretion analysis techniques to identify cells. Therefore, it is the goal for us to conduct this thesis’s research to fill in these gaps by showing the potential of using an alternative optics based approach; in particular, a system that does not require the use of fluorescent cell markers.
1.2 Importance of the Research

There are instances where flow cytometry and/or other cell-sorting techniques requiring cell markers are either undesired or unable to distinguish one cell type from another. For instance, a current clinical trial being employed for treatment of lung cancer requires that the cells be sorted so that the correct ones are being used for treatment. More specifically, T-cells are collected from blood samples taken from the patient and are genetically modified to recognize and attack the invading cancerous cells. However, the use of fluorescent markers for cell sorting, and the subsequent removal of the markers before application, results in a noticeable loss of cell viability. Therefore, there is an ever increasing need for new, unique and/or supplemental techniques and instruments that have the capacity to distinguish cells from one another on an accurate, consistent and effective scale without the need for fluorescence-based cell markers.

1.3 General Ideal of the Research

We propose that the identification of a particular cell type based on the optical force the cell experiences may have the ability to supplement, or replace, currently used cell-sorting techniques in a few specific instances, such as distinguishing cancerous cells from normal cells. Starting from the gene mutation, the development of cancer is accompanied with phenotypical changes at the cellular level including overall size, shape, internal structure, and surface membrane properties. A change in cell size or shape results in different effective scattering cross-section of the cell, and, thus, different optical force the cell experiences. In addition, changes in the internal structure or surface membrane
composition of a cell lead to the changes in its refractive index and, again, changes in the magnitude of optical force acting on the cell. Therefore, even the most subtle phenotype changes of a cell are distinguishable by evaluating the optical force generated by its interaction with a focused laser beam. By simply measuring the traveling velocities of a cell resulting from these optical forces, it is possible to distinguish phenotypically different cell types. Ultimately this exemplifies a novel and unique method of diagnosing a disease or sorting a sample of cells without the need for individual biological markers.

1.4 Research Objectives

1.4.1 Long Term Goal

The long term goal for this thesis research is to develop an optical guidance-based cell sorting device that will not rely on any cell marker to distinguish different cell types. To achieve this goal, we intend to obtain proof-of-concept data to show that different cell types guided with the same laser beam will experience different resultant optical force that is affected by many factors, including the overall size, shape, internal structure, and surface membrane properties.

1.4.2 Specific Aims

The specific aims of this research are to 1) modify, optimize, and test the laser guidance system to achieve repeatable guidance and 2) demonstrate the potential applications of laser guidance based cell sorting technique using specific examples where other methods of cell sorting are not capable of the task. The examples consisted of the following:
1. A collection of breast cancer modeling cells: normal, metastatic, and non-metastatic
2. Genetically modified lung cancer cells against their non-modified counterpart
CHAPTER TWO

LITERATURE REVIEW

2.1 Applications of Cell Sorting

Cell sorting has played a vital role in clinics and biomedical research over the years. The impact it has had on numerous research topics has been outstanding. This review will go into more detail many examples of how a variety of techniques, including the most influential, flow cytometry, have done this.

In the past, research has relied on more simplistic measures to create collections of specific cell types, included centrifugation techniques that separate cells based on density and size[11], and panning, where only particular cells attach to a surface that has been treated with a specific peptide or antibody[12], as seen in figures 2.1(a) and (b) below. Centrifugation is effective in separating cells when the cells are known to have significantly different density or size characteristics from one another. However, often cells have similar characteristics when it comes to density and size making centrifugation difficult. Panning, on the other hand, is independent from cell size, shape, and density and can cause only cells containing a specific surface protein to bind to a specifically treated surface. Again, panning reaches its limitations when there are multiple cell types in culture, many of which have the similar surface characteristics but different phenotype. These are frequently used due to their ease of application, but are limited to low levels of purity along with a tendency to have low resolution. Therefore, they are not suitable
when using small volumes of anaylate. Although not particularly useful when large, high-purity sample sizes are needed, nonetheless, they are still used in many cases.

![Centrifugal Force](image1.png) ![Panning](image2.png)

Figure 2.1 - Gravitational force based (centrifugation) (a) and panning (b) sample purification techniques.

In the early 1970’s flow cytometry was conceived. Flow cytometry is a method based on evaluating a cell’s chemical features using fluorescent markers and using that information to sort one type out from the others. The system works by first producing micron-sized droplets, each containing individual cells that have been tagged with a particular antibody/fluorescent probe. Figure 2.2(a) gives an illustration of droplet formation created by high frequency vibration of a small diameter orifice discharge nozzle. Each droplet is then passed through a focused laser beam which excited the fluorescent markers on the surface of the cell and the resulting fluorescence emissions are recorded, analyzed, and the specific fluorescent markers present can be determined. Finally, this data can be used to determine what cell type was contained in that particular droplet. Each droplet is then deflected into a specific collection chamber. Figures 2.2 (b) and (c) give a better representation of how the system essentially works. Simply put, a mixed
sample of cells is fluorescently tagged, formed into small diameter droplets, analyzed by a laser source, and deflected into a collection chamber based on a charge given to each droplet after its phenotype is determined.

Flow cytometry has been widely used in cell biology for the last 15 to 20 years where they are currently making their way from complicated intimidating devices into standard laboratory equipment. Flow cytometry, much like most histological techniques, effectively distinguishes cells from one another via specific surface antigens that a cell may express. Because of this, it can be very effective in determining the presence of specific disease causing cells[6]. It is quickly replacing fluorescence microscopy and histological techniques in the diagnosis and classification of many hematologic disorders[7, 10, 14], as well as for the evaluation of graft quality[15]. For instance, leukemia and lymphoma is frequently diagnosed with the help of flow cytometry[14].
The cell’s DNA is fluorescently labeled, excited by the laser, and the magnitude of the resulting fluorescence can be related to the quantity of chromosomes present in the nucleus and therefore determine whether they are polyploidy, an indicator of leukemia[8]. The numbers of polyploidy cells are then counted, and the resulting data gives the doctor more information to evaluate the case as a whole. This technique has also been effective in determining the stage of HIV an infected patient is in by counting the number of T-cells present in a blood sample[16]. What’s more, to study the progression of the HIV virus, it is often essential to use cell samples that have been purified of all cells other than the effected T-cells. Cell sorting has been used to efficiently do this, via the T-cell’s known expression of CD3, CD4, and CD8 receptors[16].

In biological research, separating a given heterogeneous sample of cells into purified collections of a single cell type has been an essential and increasingly important tool. For example, cells derived from the central nervous system are normally characterized by manual counting on a slide after specific immunolabeling. It was found in the literature that flow cytometry can accurately and quickly identify the number and types of neural cells present in a sample[17]. Also, flow cytometry has played a very important role in identifying and sorting mammalian germ cells (testis and sperm) for use in research related to reproductive physiology, pathology and toxicology[18]. Much more advanced techniques in cell-based therapeutic treatments require that the cells used are of high purity. For example, flow cytometry is used to create collections of a single type of cell,
in many cases hematopoietic stem cells[19], that could then be used in the treatment of liver disease[20]. These recent successes, along with its ability to sort cells at rates upwards of 25,000cells/sec with separation accuracy exceeding 90% purity[21], have led the research community to be widely accepting of the technique.

As the field of cell sorting becomes increasingly more important, many groups are beginning to search for new cell sorting techniques that address any areas where fluorescence-based techniques are not desirable. For example, one study showed there still exists a need for a mammary gland stem cell assay that could be used with flow cytometry[22]. In this particular case an alternative technique would be very useful. Also, in a small number of cases there has been documentation of the negative effects that fluorescent probes have on a cell’s viability[23, 24]. What’s more, the process of applying antigen-specific fluorescent markers is labor-intensive, time consuming and there exists the possibility of false-positive readings[25]. For this reason, there are some flow cytometry systems that are able to evaluate cells not with fluorescence but by evaluating the backscatter of light that results when the laser is scattered by each droplet/cell[26]. Thus a general live-cell analysis technique allowing detection of broad cell-based changes or differences, without additional cell manipulation, is advantageous for quantitative analysis in all types of cell based research.
2.3 Optical Force Based Techniques

There have been four significant approaches to optical force based cell sorting techniques, each of which focuses on the fact that phenotypically different cells will have unique optical properties. Finding the best way to measure those properties is the only difference among them. First to mention is optical chromatography. Much literature has been published on this topic[27]. Optical chromatography, much like traditional column chromatography, is based on having two opposing forces acting against one another, in this case the forces from microfluidic flow and a propagating laser. Assuming the cells are all of relatively the same size, the microfluidic force will be essentially equal for each cell type. However, since each cell type will have a unique set of optical properties, the force generated from the laser will be different for each cell. Therefore the cells will separate themselves into small groups while driven by the fluid as shown in figure 2.3(a).

Researchers have also established another method, recently published in Nature[28], where an array of focused laser beams was used to create an optical lattice. Essentially, the optical lattice was designed to deflect cells with one particular set of optical properties into one collection chamber and allow any other cells to go un-deflected into a second collection, illustrated in figure 2.3(c). Yet another technique has been researched where a collection of cells, while resting on the surface of a dish, are deflected by means of a rectangular cross-section laser beam scanning the area in one direction[29]. This scanning results in deflection of the cells where phenotypically different cell’s deflection measurements are unique to one another. One last study to note uses two opposing lasers that are oriented very close to one another. A very slow velocity microfluidic flow is
used to force cells into the path of these lasers where they are trapped by the optical force. In addition, the opposing forces of the lasers results in a deformation of the cell[30], figure 2.3(d). Again, much like the other techniques already mentioned, this deformation is measurable and unique to different cell types. Specific examples of how each of these techniques has been applied to research can be seen in table 2.1.

Figure 2.3 - Examples of current optics based cell sorting techniques; optical chromatography (a); optophoresis (b); microfluidic sorting in an optical lattice(c); and optical stretcher (d).

In table 2.1, a summary list of all significant methods and attempts at cell sorting are shown along with examples of how they have been applied.
Table 2.1 - Current techniques for separation of cells

<table>
<thead>
<tr>
<th>Separation Principle</th>
<th>Method</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A) Based on physical properties</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Density | Ficoll density | - Isolation of mononuclear cells from blood[31]  
- Removal of dead cells[32] |
<p>| Density | Percoll density | - Fractionation of leukocyte subsets[33] |
| Density, cell size | Elutriation | - Isolation of hematopoietic cells, separation of cells according to their cell cycle, etc.[34] |
| Electric charge | Free-flow electrophoresis | - Erythrocyte fractionation[35] |
| <strong>B) Based on immunological parameters</strong> | | |
| Ab specific protein coating on culture dish | Panning | - Useful when a specific receptor is known and its specific antibody is available.[12] |
| Specific lysis of Ab-coated cells | Complement lysis | - Depletion of an specific cell type[36] |
| Cell-cell interaction Ab-mediated | Rosseting | - Depletion of specific leukocyte subsets[37] |
| Immunoabsorption | Chromatography | - Isolation of leukocyte subsets[38] |
| Ab specificity | Avidin columns, silica particles, Ag-covered nylon | - Isolation of T-cells, leukocyte subsets, B-cells[38] |
| <strong>C) Based on biological characteristics</strong> | | |
| Osmotic shock | Hypoosmotic solutions | - Erythrocyte lysis[39] |
| Phagocytosis | Adhesion to plastics | - Removal of macrophages[40] |
| <strong>D) Based on biochemical characteristics</strong> | | |
| Inactivation of glycoprotein pump | Dye retention | - Depletion of activated T-cells[41] |</p>
<table>
<thead>
<tr>
<th>E) Based on combinations (Fluorescence)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ab specificity, magnetism</td>
<td>MACS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>F) Based on optical properties of cells and resulting optical force</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mechanical properties (viscoelasticity)</td>
<td>Optical Stretching</td>
</tr>
<tr>
<td>Size comparison</td>
<td>Cell Size in Optical Trap</td>
</tr>
<tr>
<td>Ab specificity, fluorescence, optical properties</td>
<td>Imaging-based using a multi-reservoir chip and laser tweezers</td>
</tr>
<tr>
<td>Ab specificity, fluorescence, optical properties</td>
<td>Flow cytometry using optical force switching</td>
</tr>
<tr>
<td>Optical properties (composition, size, shape, etc.)</td>
<td>Orientation of cell in an optical trap</td>
</tr>
<tr>
<td>Optical properties (composition, size, shape, etc.)</td>
<td>Optical Chromatography</td>
</tr>
<tr>
<td>Optical properties (composition, size, shape, etc.)</td>
<td>Optophoresis – Cell deflection</td>
</tr>
</tbody>
</table>
Optical properties (composition, size, shape, etc.)

Microfluidic Sorting in an Optical Lattice

- Separation of 2um silica spheres and 2um polymer spheres in a heterogeneous collection[28]
  - Separation of protein microcapsules and sorting of erythrocytes from lymphocytes[56]

2.2 Morphological Alterations in Diseased Cells

When a cell changes from a healthy phenotype into a diseased state, its physiological change will be accompanied with morphological changes as well. These changes can range from a simple alteration in the proteins that are present in the intracellular matrix to large variations in cell/organelle size. Table 2.3 exemplifies some specific examples of this.

Table 2.2 - Diseased state cell process morphological changes.

<table>
<thead>
<tr>
<th>Disease/Condition</th>
<th>Physiologic Change (example)</th>
<th>Morphological Change (optical property affected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Alcoholic liver disease</td>
<td>Enlarged Mitochondria</td>
<td>- Large, abnormally shaped mitochondria[57]</td>
</tr>
<tr>
<td>- benign tumors (salivary glands, thyroid, parathyroid, and kidneys)</td>
<td></td>
<td>- abundant, enlarged mitochondria[58, 59]</td>
</tr>
<tr>
<td>- metabolic disease of skeletal muscle</td>
<td></td>
<td>- abundant, enlarged, abnormal cristae, and contain crystalloids[60]</td>
</tr>
<tr>
<td>- cell hypertrophy and atrophy</td>
<td></td>
<td>- increase and decrease, respectively, in number of mitochondria[61]</td>
</tr>
<tr>
<td>- dysplasia and carcinoma of epithelial cells</td>
<td>Increased nucleus size</td>
<td>- nuclei become enlarged, crowded and hyperchromatic[62, 63]</td>
</tr>
<tr>
<td>- preinvasive neoplasia</td>
<td></td>
<td>- non-dysplastic nuclei (5-10um); dysplastic nuclei (~20um)[64, 65]</td>
</tr>
<tr>
<td>- genetic red blood cell disease</td>
<td>Alterations in cytoskeleton</td>
<td>- become small, sphere-shaped and fragile[66]</td>
</tr>
</tbody>
</table>
More specifically, there are more than 100 distinct types of cancer, and subtypes of tumors that can form in the human body. Each having its own respective complexities and expressing a unique set of properties that distinguishes itself from others[67, 68]. The formation of a malignant growth can be defined as the combination of 6 alterations in the cell physiology: self-sufficiency in growth signals, insensitivity to growth-inhibitory(antigrowth) signals, evasion of programmed cell death(apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis[69, 70]. Each of these physiologic changes results in many morphological deviations from normal.

Table 2.3 - Morphological alterations in cancer state cells

<table>
<thead>
<tr>
<th>Cancerous Trait</th>
<th>Physiologic change (example)</th>
<th>Morphological change (Optical property affected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Self sufficiency in growth signals</td>
<td>Acquired ability to synthesize own GFs</td>
<td>Alterations to the cell surface composition (refractive index)</td>
</tr>
<tr>
<td>Insensitivity to growth-inhibitory(antigrowth) signals</td>
<td>Overexpression of the c-Myc oncoprotein</td>
<td>Internal protein composition (refractive index)</td>
</tr>
<tr>
<td>Evasion of programmed cell death(apoptosis)</td>
<td>Underexpression of p53 tumor suppressor protein</td>
<td>Internal protein composition (refractive index)</td>
</tr>
<tr>
<td>Limitless replicative potential</td>
<td>Upregulation of telomerase enzyme</td>
<td>Internal protein composition (refractive index)</td>
</tr>
<tr>
<td>Sustained angiogenesis</td>
<td>Produce VEGF inducer</td>
<td>Internal protein composition (refractive index)</td>
</tr>
<tr>
<td>Tissue evasion and metastasis</td>
<td>Inactivate E-cadherin</td>
<td>Alterations to the cell surface composition (refractive index)</td>
</tr>
</tbody>
</table>
Consequently, these changes create unique and distinguishable variations in the optical properties of the cell. Therefore, comparing the optical properties of cells to one another has the potential to determine the presence of a morphologically altered cell “hidden” in a multi-cell collection..
CHAPTER THREE
SYSTEM THEORY

3.1 Origin of Laser Guidance

Optical forces are derived of two main components; the scattering force and the gradient force. The scattering force is created by the reflection of the traveling photons off of the surface of an object generating a momentum transfer to that object. In figure 3.1 this momentum transfer is created by the 1’ and 2’ components of the main 1 and 2 rays, respectively. This component of the force creates the force acting in the direction of laser propagation, downwards in figure 3.1 (Force components $F_{1'}$ and $F_{2'}$). The second force is created by the refraction of light when it enters the object. This gradient force is the result of the object being attracted to the highest intensity region of the beam (Force components $F_1$ and $F_2$). In the case of a focused laser beam, this force pulls the object radially into the axis of the beam as well as axially into the focal point of the beam. In order to achieve laser guidance the scattering force must be greater in magnitude than the axial component of the gradient force. If this is not the case and the axial component gradient force overwhelms the axial scattering force, then the object will be attracted to the focal point, as shown in figure 4.2, creating a point where the object becomes trapped, also known as an optical trap. In order to avoid this, a lower numerical aperture (NA) lens is used to focus the beam. When a high NA lens is used, the light will diverge quickly to a tight focus spot size and quickly expand beyond this point resulting in what was previously explained as an optical trap. When a low NA lens is used, the spot size of the beam is not only slightly larger, but is also much less convergent at the focal point.
Therefore, the gradient force in the axial direction will be much less than in an optical trap and the axial scattering force will be large enough to overcome it.

Figure 3.1 - Scattering and gradient forces created by both optical traps and laser guidance. An axial scattering force greater than the axial gradient force results in laser guidance.

3.2 Optical Force Theories

Manipulation of small neutral particles by lasers is the result of radiation forces[71]. These forces arise from the momentum of light itself. They can be large enough to accelerate[72, 73], decelerate[27], deflect[28], trap[74-76], and guide small particles.
This force is related to the intensity and the intensity gradients of the laser beams as well as the size, shape and refractive index of the particle experiencing it. Particles can range from nanometer scale (e.g. atoms and viruses) up to micrometer scale (e.g. small dielectric spheres and single living cells). These manipulation techniques give rise to major advances in fields where small particles play a major role by providing a remarkable degree of control over their dynamics, such as in cell sorting.

Theoretical models for forces of this scale and origin have thus been classified into 3 sub-categories, the Rayleigh regime, the Mie regime and the Ray Optics regime. In order to determine which method is appropriate for a given application, each of the three theories has been proven for a unique range of particle size and light wavelength.

3.2.1 Rayleigh Regime

When the particle being exposed to a focused beam of light is small relative to the wavelength of the light itself (a<< λ), the perturbation of the wavefront is minimal and the particle can be viewed as an induced dipole behaving according to simple electrostatic laws[77]. This approach is applicable when sizes are much smaller than the average living cell. For example, atoms, proteins, and viruses would fall into this category.
3.2.2 Ray Optics Regime

In the case where the particle is very large compared to the wavelength (a >> λ), the laws of geometrical optics can be used to estimate the scattering pattern due to reflection and refraction, while diffraction effects are neglected[77]. Consider a beam of parallel rays focused to a point, illuminating a single spherical particle. If the particle is large, compared to the wavelength of the incident light, the rays will be reflected and refracted at the particle surface according to Snell’s law. The intensity and phase of the rays will follow from the Fresnel coefficients. The resulting radiation force can be broken into a force parallel to the direction of the ray and orthogonal[78, 79]. However, ray optics calculations fail to address the effects arising from diffraction and are, therefore, best used to approximate forces on particles that are large scale, in this case, much larger than an average living cell.

3.2.3 Lorenz-Mie Regime

The Lorenz Mie theory (LMT) is best described as a calculation of the scattering of a plane wave by a spherical particle. LMT is fundamentally correct and valid for arbitrary particle sizes, refractive index, and wavelength. According to van de Hulst, all problems in theoretical optics are problems in Maxwell’s theory and should be treated as such when a full, formal solution is required[80]. The solution to Maxwell’s equations with the appropriate boundary conditions is required for a full arbitrary theory. The first step in that direction was taken in the beginning of the 20\textsuperscript{th} century when Mie[81] and Debye[82], building on earlier work by Lorenz[83], each presented their own solutions
for the scattering of a plane wave by a spherical particle. However, an infinite plane wave is only an approximation of light emanating from a distant source, and so cannot describe a Gaussian beam, such as that produced by a TEM$_{00}$ mode laser.

Therefore, the Generalized Lorenz Mie Theory (GLMT) was proposed. Generalization of the LMT has been presented by many researchers through theoretical and some numerical results[84, 85]. The most extensive work was presented by G. Gouesbet et. Al. and is termed the Generalized LMT (GLMT)[86]. GLMT introduces an infinite set of beam-shape coefficients as a partial wave expansion to describe the non-plane wave nature of the illuminating beam. These beam shape coefficients can be resolved with reasonable speed using the improved localized approximation[87], which has been justified rigorously for the case of Gaussian beams[88], and more recently for arbitrary shaped beams [89]. In addition, GLMT has been successfully applied to cylinders [90] and multi-layered spheres[91].

Scattering forces in GLMT are given by:

$$\vec{F}(\vec{r}) = \left(\frac{n_m}{c}\right)\frac{2P}{\pi\omega_0^2} \left[\hat{x}C_{pr,x}(\vec{r}) + \hat{y}C_{pr,y}(\vec{r}) + \hat{z}C_{pr,z}(\vec{r})\right]$$

(3.1)

Where:

$C_{pr,x}, C_{pr,y}, and C_{pr,z}$ are the cross sections for radiation pressure as presented in the original work of Gouesbet et al.[86].

In contrast to ray optics ($a>>\lambda$), GLMT can predict the presence of resonance, signifying the creation of electric and magnetic multi-poles in the particle. Resonance effects cause
fluctuations in the trapping forces as a function of the wavelength and particle size; their detection requires proceeding in very small steps in the size parameter \( \alpha = \frac{2\pi i}{\lambda} \).

GLMT results have been previously compared with optical levitation experiments and were found to be in agreement[92, 93].

3.3 Laser Types and Mode Comparison

To obtain optimal experimental conditions, it is crucial to have an ideal beam profile throughout the design. Beam quality is a factor of multiple considerations including laser output mode, the surrounding atmospheric environment, and the optical manipulation of the beam. First, we must consider the output of the laser source. In general, laser-beam propagation can be approximated by assuming that the laser beam has an ideal Gaussian intensity profile, corresponding to the theoretical TEM\(_{00}\) mode. Unfortunately, real-life laser sources do not produce a truly ideal Gaussian profile, although many, such as helium neon, argon-ion, and Ti:Sapphire, come very close. Therefore, a distinct value has to be determined for every laser source that corrects for this variance from an ideal profile. This value is termed the quality factor, \( M^2 \) (also known as the “M-square” factor). In the case of an ideal Gaussian profile this \( M^2 \) value is equal to 1 and anything varying from ideal has a value greater than 1. For example, a helium neon laser typically has an \( M^2 \) value less than 1.1, whereas, a multi-mode diode laser can have \( M^2 \) values as high as 25 or 30.

The mode of the laser beam can be defined in cylindrical form in terms of radius \( (\rho) \) and angle \( (\varphi) \). The eigenmodes \( (E_{\rho \varphi}) \) for this equation are a series of axially symmetric
modes. For stable resonators, the modes are approximated by the Laguerre-Gaussian functions, denoted by $\text{TEM}_{\rho \varphi}$.

It is noticed from this figure that there is a large variation between the $\text{TEM}_{00}$ and $\text{TEM}_{01}$ beams. Therefore, specifying the mode in this form is inadequate, because, for example, the output of a laser can contain up to 50% higher modes and still be considered $\text{TEM}_{00}$. For that reason, it is necessary to define the beam propagation mode numerically.

With $M^2$ defined, it is now possible to more accurately determine the propagation characteristics of a real beam, as well as apply it to modify the lens equation (see Appendix F).

The particular $M^2$ value was not defined in this research; however, it is important to understand the difference between the many laser sources available. More importantly, that their qualities will vary, therefore affecting the quality of the assumptions made in many of the following results.
3.4 Cell Viability

When working with any focused beam of energy, it is important to ensure that there is no photothermal damage to the exposed cells. Therefore, previous research in our lab was done to ensure that there was no negative effect that could compromise the viability of the cells post sorting. Studies measuring cell growth and DNA damage, via a modified COMET assay, were conducted at varying energy intensities and time durations far exceeding the range necessary to characterize a cell type using our technique. The results showed no significant difference between the cell growth potential, as well as no significant damage to the cellular DNA when compared to unexposed cells[94].
CHAPTER FOUR
EXPERIMENTAL DESIGN

The design, construction and optimization of the system can be most easily categorized into the following:

1. Laser source and beam characteristics
2. Optics setup
3. Guidance chamber
4. Imaging and Illumination

4.1 Laser Source and Beam Characteristics

A TEM$_{00}$ mode, tunable (750nm – 950nm), CW (continuous wave), Nd:YVO$_4$ pumped, solid state Ti:Sapphire laser (3900S, Spectra Physics) was used as the laser source for the following research. This, along with all of the following components of the system, was mounted onto a vibration isolation breadboard table (RS 3000, Newport) that maintained a level surface using pressurized nitrogen gas.

4.1.1 Power

The power and intensity of the laser is of utmost concern when dealing with any biological material. Excessive thermal conditions have the potential to negatively affect the viability of the cells[94]. From experimental trial and error, it was found that optimal power for guidance of particles was 50mW and 175mW for guidance of cells. These
powers were used for each respective experiment to maintain consistent experimental conditions.

4.1.2 Wavelength

Previous work by Svoboda and Block[84], as shown in figure 4.1, provides an optimal range of wavelength to use when biological specimens are used. They show that cellular material, such as hemoglobin and cytochrome, absorbs most readily at lower wavelengths, such as that in the visible region of the spectrum. At longer wavelengths, infrared, the suspension solution absorbs much of the energy. Therefore, it is between these ranges that this research is conducted (approximately 750nm-1250nm).

![Absorption curve for Biological material and water versus wavelength.
Obtained from Svoboda and Block[87]. (Hb and HbO2 are deoxyhemoglobin and oxyhemoglobin, respectively)](image)

A spectrophotometer (USB 2000, Ocean Optics) was used to monitor the wavelength of the emitted light which was set at a wavelength of 800nm for all experiments. This
wavelength, as well as the above mentioned powers, have also been previously used and were successful in biological application[76, 95-100].

4.1.3 Waist size

The waist for laser guidance is focused in such a way that it has a longer focal region with a larger waist size (slightly smaller than the size of the average cell). This provides a smaller downward acting gradient force that can be overcome by the upward scattering force, ultimately leading to a total force acting in the upward axial direction, as shown in figure 4.2. Also, it is desirable and convenient for the design of the system to have a larger working distance, which a less tightly focused beam provides as well.

![Figure 4.2 - Comparison between the beam characteristics of laser guidance and laser trapping forces.](image)

In order to determine what waist size would achieve laser guidance as explained above a series of theoretical calculations using the GLMT software were done. The conditions
for the theoretical calculations were held as they were planned for the actual experiments (wavelength: 800nm; Power: 175mW; PBS Refractive Index: 1.33;). Also necessary was to estimate the cell size and refractive index. Although these parameters are difficult to quantify accurately as they are different for diverse cell types, it was possible to estimate their values based on some averages found in the literature. Therefore, the refractive index for cells was estimated to be 1.36 and their size to be approximately 10um (the general estimate of the average cell). Using these values the only parameter left to vary was the waist size of the focused laser.

In figures 4.3 - 4.5, three theoretical axial force profiles are shown with three different waist size values. In figure 4.3, where the waist size is 2.5um, there is a region of the graph where the axial force is a negative value. This point represents where the gradient force (acting opposite laser propagation) is overcoming the scattering force (acting with laser propagation). At this point the resultant total force is therefore acting against laser guidance and the cell would theoretically begin moving in the opposite direction. This eventually leads to a “teeter tottering” back and forth at this point and finally the cell will come to an equilibrium where the total net force is zero and becomes trapped, also known as an optical trap. In figure 4.4, with a 3.0um waist size, there are no negative forces, but the force approaches very close to zero within one region. Within this region there would be no force acting on the cell and therefore zero velocity as well. Finally, in figure 4.5 the waist size is slightly larger (3.5um) and the force is greater than zero for all regions. This represents a total positive force (acting with laser propagation) that results in laser
guidance at all points along laser propagation as desirable for this research. Therefore, this tells us that in order to achieve laser guidance at all points along laser propagation the waist size must be greater than 3.0um. However, it is also desirable to keep the waist size relatively small. When the waist size becomes much larger than the cell being guided, much of the force from the laser will not be transmitted to the cell. For this reason, it is recommended to set the waist size to just larger than the minimum value.

Figure 4.3 – Axial force created by a focused laser beam with a waist size of 2.5um resulting in a negative force creating a trapping point
Figure 4.4 – Axial force created by a focused laser beam with a waist size of 3.0 um resulting in a region of zero force.

Figure 4.5 – Axial force created by a focused laser beam with a waist size of 3.5 um resulting in a positive (guiding) force in all regions.
At last, a 60mm lens (NA = 0.27) was shown to focus the laser to a waist size greater than 3.0um, which also provides a practical amount of space for other component installation. The actual waist size was measured and the results are explained in the results section of this thesis.

4.1.4 Gaussian Fit

Although the design specifications of the laser claim a TEM$_{00}$ mode, there are still other factors that could produce undesirable effects on the quality of the beam. A BeamMaster-3 digital knife-edge laser scanner (BM-3, Coherent Inc.) was used to characterized the laser quality and determine the reliability of a perfectly Gaussian beam profile.
Figure 4.6 - Screen shot of BeamMaster-3 digital knife edge laser scanner measurement of Gaussian fit of laser.

Although the results did not claim a perfect profile, the experimental results stated in this research depend mostly on relative calculations. Therefore, as long as the beam quality was constant for all experiments, the results would not be significantly affected.

4.2 Optics Setup

In order to obtain optimal beam characteristics within the guidance chamber, it is necessary to properly align the laser as it propagates to that point. It is first required to confirm that the laser is propagating on a parallel path with reference to the balance table. To do so, a series of irises were mounted to the table and height measurements were
taken. Once this was accomplished, the actual optics were mounted and properly aligned. Since the spot size of the laser being focusing inside the chamber is dependent on both the focal length of the lens and the input diameter of the beam into that lens, it was desirable to alter the output size of the beam from the source to a magnified size. This beam expansion setup plays a secondary role in the filtering of undesirable spatial noise that the beam may contain. This noise may be due to the reduced quality of the beam emitted from the source or simple diffraction of the beam as it propagates through free space. By focusing the beam through a small pinhole (25µm), this noise can be removed resulting in a more ideal Gaussian profile. Figure 3 shows in detail the optics setup used to accomplish this. The beam is first passed through a 20mm focusing lens, followed by the 25µm pinhole. Micromanipulators were used to reposition the optics in order to pass the maximum intensity of light through the pinhole. The expanding beam is then “collected” using a 75mm plano-convex lens producing a collimated beam that has been expanded by a factor of 3.75. The beam then propagates towards a prism.

Figure 4.7 - Beam expansion and noise filter lens setup
This prism redirects the propagation of the beam vertically towards the final focusing lens. Again, a 60mm (NA=0.27) plano-convex lens was used to focus the beam into the guidance chamber, where velocity measurements were taken.

Figure 4.8 - Basic schematic of system setup
Figure 4.9 - System components and experimental setup
4.3 Guidance Chamber

The guidance chamber had to be carefully designed to ensure that the results were conducted in the most ideal conditions possible. There are 3 major design concerns:

1. Minimize convective forces
2. Liquid tight
3. Imaging windows

In order to obtain accurate velocity measurements, any undesirable background forces need to be minimized. In the case of laser guidance, convective forces created by temperature gradients in the fluid are most significant. When the laser propagates through the suspension media, the laser acts as a heat source that can heat the fluid body, ultimately creating eddies within the chamber that disrupts the once stationary fluid. To minimize this effect, the size of the chamber must be kept to a very small size while still maintaining functionality and ease of manufacture. When the chamber size is small, it is much more difficult for large temperature gradients to emerge, therefore producing minimal convection. On the other hand, making the chamber too small can result in partial blockage of the laser entering the chamber as shown in figure 4.7. The laser diverges out from the waist location quickly and reaches ~120um in diameter just 2.5mm from the waist and doubling in size every mm after that. Therefore, when guidance was being measured in the middle of a 10mm tall chamber, the minimum window width in order to not block any of the laser would have to be ~720um. This calculation along with
the limitations of the fabrication method resulted in the chamber having a 2mm x 2mm cross section and 10mm in height.

Next, since the polymer material of the chamber was porous relative to the fluid, it was necessary to coat the chamber with a layer of PDMS. This coat, along with the use of room-temperature curing silicone sealant, produced watertight conditions. Finally, #1 8mm x 8mm cover glass slips were used to enclose the guidance region. This allowed for side-on imaging as well as propagation of the laser entering at the bottom and exiting through a window on the top.
4.4 Imaging and Illumination

An illumination source provides light through one side of the chamber, while an imaging setup is used to capture the guidance occurrences on the other. A 10X long working distance microscope objective is connected to a high resolution CCD camera, with multiple IR filter lenses inline to reduce saturation of the images from any unwanted IR light that passes through the objective. A computer equipped with a Matrox Meteor II
frame grabber captures images from the camera at a rate of 30 frames/sec and a resolution of 768 x 494. The images are recorded using Video Savant 4.0 software.
5.1 Waist Size Determination

To accurately measure the waist size of the focused beam, the knife-edge technique was used. This measurement was used for two reasons. First, the value is necessary for use in the theoretical calculations to predict the guidance events before the actual experimentation, and second it is a good reference when optimizing the system design. Knife-edge technique combines the measurement of changes in beam power as a sharp knife edge cuts its. These measurements, along with a series of mathematical integrations, can be used to determine the $1/e^2$ waist size of a beam (figure 4.1 and 4.2).

![Figure 5.1 - Profile of a Gaussian beam with the $1/e^2$ beam waist parameter](image-url)
Using a series of these scans above and below the waist of the beam, an estimation of the smallest spot size can be determined (see Appendix G). A BeamMaster-3 (Coherent Inc.) 3-blade digital knife-edge scanner was used to collect multiple scans of the beam.
Using this data and equation 1, the waist size can be approximated.

\[ w(z) = w_0 \sqrt{1 + \frac{\lambda(z - z_0)}{\pi w_0^2}} \]  

(5.1)

Where:
- \( W_0 \) = Waist spot size (m)
- \( \lambda \) = Wavelength (m)
- \( z_0 \) = Axial position of waist (m)
- \( z \) = Axial position of interest (m)

5.2 Laser Guidance of Particles

8.31\( \mu \)m and 10.2\( \mu \)m sphere-shaped particles of refractive index 1.56 were used for calibration and proof of theory experiments. The particles were each suspended in a 20 to 1 ratio of PBS and particles, respectively. For each experiment the chamber and injection fiber were first flushed with distilled water 3 times, followed by flushing with PBS 3 times. The glass portion of the chamber was then filled with approximately 5mm of PBS and the chamber top was sealed with a PDMS gasket and 4 screws. A 50\( \mu \)L syringe was filled with the particle/PBS solution and attached to the microinjection fiber. The laser was set to 800nm wavelength and a power setting of 40mW, at the chamber. Approximately 10\( \mu \)L of suspension was injected into the chamber and recording was started on the VideoSavant software. After multiple guidance occurrences were captured, the images were saved for further analysis.
5.3 Laser Guidance of Cells

The protocol for all cell types was identical. For each cell type, trypsin was added to the T-75 flask for ~10min. The cells were collected in a 10mL centrifuge tube and neutralized with a volume of media equal to that of the trypsin used. The cells were then spun down using centrifugation force of 1000rpm for a duration of 8 minutes. The pellet of cells that resulted was then resuspended in 2mL of PBS. A 50µL syringe was filled with the cell/PBS solution and attached to the microinjection fiber. The chamber, after being exposed to UV radiation for sterilization of ~24 hours, was filled with ~5mm of PBS. The laser was set to 800nm and 125mW, at the chamber. 10µL of the cell suspension was injected into the chamber and VideoSavant recording was started. After collection of multiple guidance occurrences of cells, the images were saved for further analysis.

5.4 Determining Velocity of Guidance

It has been previously found with a 10x objective the image size is 756x484 pixels, or one pixel = 0.929 microns. Therefore, we image approximately a 450µm portion of the beam. The position of the field of view of the objective is adjusted initially to view the region around the beam waist. It is at the waist location that the maximum velocity of guidance can be obtained. The process of converting the captured images to a velocity measurement is carried out using MatLab programming software. When a particle enters the field of view that frame is taken as the first frame. Once the particle is no longer visible in the field of view, the last frame is the taken as the last position of the particle
inside the field of view. From each image, the particle/cell being guided is selected, and a series of steps involving ‘selecting and cropping’ of the particle/cell, threshold, and edge detection, followed by centroid calculation is done to determine the location of the particle/cell in each image. We have been able to successfully use this method to determine the centroid of both the polystyrene microspheres and cells that were guided by the laser beam. From the steps shown in figure 4.4, the distance moved by the particle/cell can be calculated as the difference in the value of particle location (centroid pixels) between 2 consecutive frames. From the knowledge of the distance moved by the particle from one frame to the next, and the frame rate of 30 frames/sec; the velocity of motion is computed as:

\[
\text{Velocity} (m/s) = \frac{\text{distance}}{\text{time}}
\]

\[\text{Velocity}\_{\frac{\text{mm}}{s}} = \frac{\text{Centroid}(x, y)_{n+1} - \text{Centroid}(x, y)_{n}}{2} \times (0.929 \mu m/\text{pixel}) \times 30(\text{frames}/s)\]

\[\text{(5.3)}\]
5.4 Steps Involved in Image processing. Position of cell in a given frame is based on calculation of centroid using “Canny” function in Matlab.

5.5 Cell Size Comparison

Cells were plated on a glass bottom 30mm round dish and immediately observed under a Zeiss microscope at 63x magnification. Multiple cells of each phenotype were imaged and captured. Using Zeiss software the diameters of each cell was measured. The resulting average cell diameters of each phenotype were then compared to one another and statistical analysis was performed.

5.6 Cell Culture

7F2, 3T3-L1, 4T1, 4T07, NMuMG, TC-1 and L-10 cell lines (American Type Culture Collection, ATCC) were cultured according to distributor’s protocol.
7F2

7F2 are mouse bone marrow derived osteoblast cells. The cell line was maintained in alpha minimum essential medium with 2mM L-glutamine and 1mM sodium pyruvate, without ribonucleosides and deoxyribonucleosides, and 500mL was supplemented with 50mL of fetal bovine serum.

3T3-L1

3T3-L1 cells are mouse fibroblasts derived from the embryo. The cell line was maintained in Dulbecco’s modified Eagle’s medium with 4mM L-glutamine, and 500mL was supplemented with 0.75g of sodium bicarbonate, 2.25g of glucose and 50mL of fetal bovine serum.

4T1

4T1 cells are mouse, metastatic mammary gland cancer cells. The cell line was maintained in Dulbecco’s modified Eagle’s medium with 2mM L-glutamine, and 500mL was supplemented with 0.75g of sodium bicarbonate, 2.25g of glucose and 50mL of fetal bovine serum.

4T07

4T07 cells are mouse, non-metastatic mammary gland cancer cells. The cell line was maintained in medium identical to the 4T1 medium detailed above.
NMuMG

NMuMG cells are mouse, non-cancerous epithelial cells derived from the mammary gland. The cell line was maintained in Dulbecco’s modified Eagle’s medium (ATCC) and 500mL was supplemented with 2.25g of glucose, 5mg of bovine insulin (Sigma), 50mL of fetal bovine serum, 5mL antibiotic/antimycotic (AA) (Invitrogen), and 1mL fungizone (Invitrogen).

TC-1

TC-1 cells are mouse, lung tissue cancer cells. The cell line was maintained in RPMI (Mediatech), non-essential amino acids (Hyclone), 100mM sodium pyruvate, 10mg/ml gentamicin sulfate (Cambrex) and 50ml of fetal bovine serum.

L-10

L-10 cells are TC-1 cells transfected with a plasmid containing a fusion protein (MULT1 extracellular and FAS(death receptor) transmembrane and intracellular region). The cell line was maintained using medium identical to the TC-1 media described above supplemented with 100mg/ml of zeocin (Invitrogen).

5.7 Cell Proliferation

The above mentioned cells were seeded (see appendix A) from frozen collections, cultured in T-75 tissue culture polystyrene flasks, allowed to proliferate to 100%
confluence and passaged (see appendix B) at least once before use. The culture medium was replaced every 48-72 hours.

5.8 Statistical Analysis

For all studies where statistical analysis was necessary, SAS® statistical software (SAS Institute, Cary, NC) was used. Sample size was maintained at \( n \geq 5 \). One-factor analysis of variance (ANOVA) was performed and values are presented as mean ± standard error of mean. The significance level for all comparisons was \( p \leq 0.05 \).
CHAPTER SIX

RESULTS

6.1 Waist Determination

In order to achieve laser guidance there was only one region of the focused laser where this was possible, starting approximately \(225\)um on one side of the beam waist and continuing past the waist another \(225\)um. Therefore, all experiments were carried out at this waist point. A knife-edge based beam profiler (Coherent Optics, Beam Master) was used to approximate the size of this spot by taking multiple measurements of the cross-sectional area of the beam at many different locations above and below the predicted location of the waist.

Figure 6.1 - Locations of beam cross-section measurements
Table 6.1 – BeamMaster knife edge scan cross-sectional diameters

<table>
<thead>
<tr>
<th>Scan Position</th>
<th>z (mm)</th>
<th>w (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6.5</td>
<td>118.74</td>
</tr>
<tr>
<td>2</td>
<td>7.0</td>
<td>95.55</td>
</tr>
<tr>
<td>3</td>
<td>8.7</td>
<td>33.85</td>
</tr>
<tr>
<td>4</td>
<td>9.3</td>
<td>58.32</td>
</tr>
<tr>
<td>5</td>
<td>9.6</td>
<td>79.24</td>
</tr>
<tr>
<td>6</td>
<td>10.0</td>
<td>104.00</td>
</tr>
<tr>
<td>7</td>
<td>10.3</td>
<td>123.62</td>
</tr>
</tbody>
</table>

These data points were then used along with equation 5.1. From these calculations, the waist size was calculated to be 3.2 µm in diameter.

6.2 Theoretical Particle Guidance

Using this waist size the force that the laser exerts on a particle that has known physical characteristics could be predicted using the GLMT simulation software. For these comparison experiments, 8.31µm ± 0.1µm and 10.2 µm ± 0.1µm diameter polystyrene particles were used. These particles also have defined refractive indices of 1.56. Using this, along with the characteristics of the laser, the force was calculated and converted to a velocity value using Stokes law of drag force.

Table 6.2 - System parameters for theoretical calculations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle Diameter</td>
<td>8.31µm ± 0.1µm/10.2 µm ± 0.1µm</td>
</tr>
<tr>
<td>Waist Size</td>
<td>3.2 µm</td>
</tr>
<tr>
<td>Particle Refractive Index</td>
<td>1.56</td>
</tr>
<tr>
<td>Suspension Refractive Index</td>
<td>1.33</td>
</tr>
<tr>
<td>Laser Wavelength</td>
<td>800nm</td>
</tr>
<tr>
<td>Laser Power (@ Chamber)</td>
<td>50mW</td>
</tr>
</tbody>
</table>
6.3 Experimental Particle Guidance

Actual guidance events using the 8.31µm and 10.2µm particles were recorded and the velocity profiles were graphed.

Table 6.3 - System parameters for particle experimentation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle Diameter</td>
<td>8.31µm ± 0.1µm/10.2 µm ± 0.1µm</td>
</tr>
<tr>
<td>Waist Size</td>
<td>3.2 µm</td>
</tr>
<tr>
<td>Particle Refractive Index</td>
<td>1.56</td>
</tr>
<tr>
<td>Suspension Refractive Index</td>
<td>1.33 (PBS)</td>
</tr>
<tr>
<td>Laser Wavelength</td>
<td>800nm</td>
</tr>
<tr>
<td>Laser Power (@ Chamber)</td>
<td>50mW</td>
</tr>
</tbody>
</table>

Theoretical and Experimental Velocity Profiles of 8.31µm and 10.2µm Particles

Figure 6.2 – Theoretical and experimental velocity profiles of 8.31µm and 10.2µm particles at a laser power of 50mW
6.4 Visual Cell Comparison

Each of the cell types used in the cancer diagnostics and genetically modified cell sorting studies were examined under a high magnification microscope. Their relative sizes were then compared to one another. In the cancer diagnostic study, pictures of the 4T1 and 4T07 cells were taken under 63X magnification (Zeiss Microscope). Multiple cells of each phenotype were observed immediately (<10min) after being plated in a glass bottomed 30mm petri dish and the data was compared.

![Figure 6.3 - 4T1 (a) and 4T07 (b) cells immediately after seeding (63X magnification)](image)

Figure 6.3 - 4T1 (a) and 4T07 (b) cells immediately after seeding (63X magnification)

![Average Cell Diameter of Breast Cancer Cells Immediately after Plating](image)

**Figure 6.4 - 4T1 and 4T07 average cell diameter immediately after seeding (p < 0.05)**
For the genetically modified cell sorting study each cell type was seeded onto a glass bottom Petri dish and viewed immediately under 63X magnification.

Figure 6.5 - TC-1 (a) and L-10 (b) cells immediately after seeding (63X magnification)

![TC-1 and L-10 Cells Immediately After Seeding](image)

Average Cell Diameter of Breast Cancer Cells Immediately after Plating

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Average Cell Diameter (um, 63x)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-10</td>
<td>12</td>
</tr>
<tr>
<td>TC-1</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 6.6 - TC-1 and L-10 average cell diameter immediately after seeding (p < 0.05)

6.5 Cell Sorting

6.5.1 Sample Purification

A suspension of media containing both osteoblast cells and debris (dead cells, cell fragments, and/or media impurities) was sorted utilizing the laser guidance system. The
fragments were visibly different from the cells and therefore were easily distinguished from the healthy cells. Figure 5.8 compares the cell and debris maximum velocities to one another.

Table 6.4 - System parameters for cellular guidance of osteoblast cell sample purification

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beam Waist Size</td>
<td>3.2 µm</td>
</tr>
<tr>
<td>Suspension Refractive Index</td>
<td>1.33 (PBS)</td>
</tr>
<tr>
<td>Laser Wavelength</td>
<td>800nm</td>
</tr>
<tr>
<td>Laser Power (@ Chamber)</td>
<td>175mW</td>
</tr>
</tbody>
</table>

![Average Maximum Velocities of Osteoblast Cells and Suspension Debris](image)

Figure 6.7 - Average maximum velocities of osteoblast cells and debris (p < 0.05)

The velocities of phenotypically different cell types were defined in another study. Osteoblasts and 3T3 fibroblasts were guided under the following beam characteristics:

Table 6.5 - System parameters for cellular guidance of osteoblast and 3T3 fibroblast cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beam Waist Size</td>
<td>3.2 µm</td>
</tr>
<tr>
<td>Suspension Refractive Index</td>
<td>1.33 (PBS)</td>
</tr>
<tr>
<td>Laser Wavelength</td>
<td>800nm</td>
</tr>
<tr>
<td>Laser Power (@ Chamber)</td>
<td>175mW</td>
</tr>
</tbody>
</table>
The maximum velocity of each cell was measured at the waist of the beam and the average values are shown in figure 5.9.

![Average Maximum Velocity of 3T3 Fibroblasts and Osteoblasts](image)

Figure 6.8 - Average maximum velocities of 3T3 fibroblast and osteoblast cells
(± st dev, p<0.05)

A second analysis was done where the average velocity over the entire guidance event, approximately 450µm, was determined for each cell type and again compared to one another.
Figure 6.9 – Average velocity of 3T3 fibroblast and osteoblast cells over approximately 450µm of guidance (± st dev, p<0.05)

6.5.2 Cancer Diagnostics

The velocities of 4T1, 4T07, and NMuMG cells were defined under the following parameters:

Table 6.6 - System parameters for cellular guidance of 4T1, 4T07 and NMuMG cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Size</td>
<td>10.05 µm ± 0.6µm (4T1)</td>
</tr>
<tr>
<td></td>
<td>9.75 µm ± 0.7µm (4T07)</td>
</tr>
<tr>
<td></td>
<td>NMuMG not measured</td>
</tr>
<tr>
<td>Beam Waist Size</td>
<td>3.2 µm</td>
</tr>
<tr>
<td>Suspension Refractive Index</td>
<td>1.33 (PBS)</td>
</tr>
<tr>
<td>Laser Wavelength</td>
<td>800nm</td>
</tr>
<tr>
<td>Laser Power (@ Chamber)</td>
<td>175mW</td>
</tr>
</tbody>
</table>

Multiple experiments for each cell type, with identical system parameters, were performed. Their velocity profiles were examined and the maximum velocity, located at the waist of the beam, was recorded and compared to one another.
A second analysis was done where the average velocity over the entire guidance event, approximately 450µm, was determined for each cell type and again compared to one another.

Figure 6.11 – Average velocity of 4T1, 4T07, and NMuMG breast cancer cells over approximately 450µm of guidance (± st dev, p<0.05)
6.5.3 Genetically Modified Cell Sorting

To show the potential of the system to sort cells based on small changes in its genetic code, both TC-1 cells and their genetically modified counterpart, L-10 cells, were examined. The cells were guided under the following conditions and their maximum velocities were recorded and compared to one another.

Table 6.7 - System parameters for TC-1 and genetically modified TC-1 (L-10) cell guidance

<table>
<thead>
<tr>
<th>Cell Size</th>
<th>11.47 µm ± 0.7µm (TC-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11.37 µm ± 1.1µm (L-10)</td>
</tr>
<tr>
<td>Beam Waist Size</td>
<td>3.2 µm</td>
</tr>
<tr>
<td>Suspension Refractive Index</td>
<td>1.33 (PBS)</td>
</tr>
<tr>
<td>Laser Wavelength</td>
<td>800nm</td>
</tr>
<tr>
<td>Laser Power (@ Chamber)</td>
<td>175mW</td>
</tr>
</tbody>
</table>

![Graph showing average maximum velocities of TC-1 and genetically modified TC-1 cells](image)

Figure 6.12 - Average maximum velocities of TC-1 and genetically modified TC-1 cells (L-10 cells) (± st dev, p<0.05)
A second analysis was done where the average velocity over the entire guidance event, approximately 450µm, was determined for each cell type and again compared to one another.

![Average Velocity Over 450µm of TC-1 and Genetically Modified TC-1 (L-10) Cells](image)

**Figure 6.13** – Average velocity of TC-1 and genetically modified TC-1 (L-10) cells over approximately 450µm of guidance (± st dev, p<0.05)

### 6.6 System Precision

The systems ability to produce consistent and reproducible results within a single cell type was determined by guiding one particular cell line on multiple occasions. This was done for both the cancer diagnostics study as well as within the genetically modified cell study. In figures 6.14 -6.17 the different bars within each cell type represent experiments conducted on different days.
Average Maximum Velocity of Various Breast Cancer Cell Phenotypes

Figure 6.14 - Average maximum velocities (± st dev) of NmuMG, 4T1 and 4T07 cells [Numbers within each bar represents sample number (N), p<0.05]

Average Velocity over 450um of Various Breast Cancer Cell Phenotypes

Figure 6.15 - Average velocity over 450um of guidance (± st dev) of NmuMG, 4T1 and 4T07 cells [Numbers within each bar represents sample number (N), p<0.05]
Figure 6.16 - Average maximum velocities (± st dev) of L-10 and TC-1 cells [Numbers within each bar represents sample number (N), p<0.05]

Figure 6.17 - Average velocity over 450um of guidance (± st dev) of L-10 and TC-1 cells [Numbers within each bar represents sample number (N), p<0.05]
Table 6.8 - System parameters for cellular guidance of 4T1, 4T07, TC-1 and L-10 cells to measure system precision

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beam Waist Size</td>
<td>3.2 µm</td>
</tr>
<tr>
<td>Suspension Refractive Index</td>
<td>1.33 (PBS)</td>
</tr>
<tr>
<td>Laser Wavelength</td>
<td>800nm</td>
</tr>
<tr>
<td>Laser Power (@ Chamber)</td>
<td>175mW</td>
</tr>
</tbody>
</table>

Figure 6.18 - Repeatability of 4T1 cell type using average maximum velocity (± st dev, p<0.05)

Figure 6.19 - Repeatability of 4T1 cell type using average velocity over 450µm of guidance (± st dev, p<0.05)
Figure 6.20 - Repeatability of 4T07 cell type using average maximum velocity (± st dev, p<0.05)

Figure 6.21 - Repeatability of 4T07 cell type using average velocity over 450um of guidance (± st dev, p<0.05)
System Precision Using TC-1 Cells and the Average Maximum Velocity

![Graph showing average maximum velocity comparison between TC-1 Experiment 1 and TC-1 Experiment 2.]

Figure 6.22 - Repeatability of TC-1 cell type using average maximum velocity (± st dev, p<0.05)

System Precision Using TC-1 Cells and the Average Velocity Over 450um

![Graph showing average velocity over 450um comparison between TC-1 Experiment 1 and TC-1 Experiment 2.]

Figure 6.23 - Repeatability of TC-1 cell type using average velocity over 450um of guidance (± st dev, p<0.05)
Figure 6.24 - Repeatability of L-10 cell type using average maximum velocity
(± st dev, p<0.05)

Figure 6.25 - Repeatability of L-10 cell type using average velocity over 450um of
guidance (± st dev, p<0.05)
CHAPTER SEVEN

DISCUSSION

Like other groups approaching cell sorting with an optical force basis, the ultimate goal of this research was to propose an efficient way to distinguish different cells from one another without the need for fluorescent tagging. It has been proposed in the past that laser guidance has this ability. It was the purpose of this study to not only show that this could be done with morphologically different cell types, but more importantly that it could be done with cell types with nearly identical morphologies but having some form of molecular alteration such as a simple modification in the genetic code. It is important to do this for two reasons. 1) GLMT calculations have shown that there is a resonance effect that plays an undesirable role when interpreting the value of a cell’s velocity when different cell sizes are used and 2) other techniques are unable to do this without the use of fluorescent markers. The resonance effect is a result of the laser interacting with different size cells in different and unpredictable ways. For example, an 8.2um cell may guide slower than an 8.3 um cell, but an 8.25um cell may guide faster than both. Basically, it is a nonlinear, almost sinusoidal, relationship that can result in unreliable data when cells of different size are studied. Therefore, this study focuses on two very specific cases where the cell types being compared are of significantly similar size, but slightly different molecular properties.
7.1 System Optimization

A major concern with the previous system design was the presence of noticeable convective forces within the chamber that resulted in less than ideal conditions for calculating reliable guidance velocities. The new chamber designed for these studies proved to be very effective in minimizing these forces. Further detail of how this was accomplished was explained in the system design section of this thesis. With the exception of this major change, the system remained relatively similar to the prior design. Once the system was assembled experiments were conducted where the velocity was measured at the focal point of the focused laser where guidance created a velocity in the range of 10 to 100 µm/sec. It was necessary to approximate the size of this focal point (3.2um) so that it could be used as an input into the GLMT software. Using this software, and the known properties of 8.31um and 10.2um polystyrene particles, the guidance velocities were modeled. These theoretical values were then compared to the actual experimental guidance of the same particles. It can clearly be seen from figure 5.3 that the profiles of each did not match up very closely. This could be the result of a combination of many different design characteristics:

- A non-Gaussian beam profile
- Slight alignment discrepancies
- Resonance effect due to varying particle sizes

Of most significance once again is this resonance effect. Therefore, knowing that it is difficult to use GLMT theory with particles, it is without a doubt difficult to use when cells are in question. Not only will the issues seen with particles be present, but there is
the additional weight of a lack of information of a cell’s refractive index. This is a very
difficult property to measure due to cells having three main, and optically different,
regions within it:

• outer cell membrane
• intracellular matrix
• nuclear region

Therefore, this makes the cell’s refractive index as a whole unpredictable. Although this
is a setback in the modeling of the system, it is ultimately the basis of how the system is
able to distinguish cells with such high accuracy. While GLMT is not an excellent tool in
predicting the specific guidance velocities of particles and cells, it is still a good tool to
show that changing specific components or parameters of a system does indeed result in a
change in guidance velocity.

7.2 Visual Cell Comparison

Next, it was important to confirm that the cells were indeed of the same size.
Furthermore, if there was a noticeable difference in cell morphology between
phenotypes, then laser guidance, or any further analysis technique for that matter, would
not be necessary, at least for diagnostic purposes. The most apparent way to accomplish
this was to simply examine the cells under high magnification. Multiple images of each
cell type from both the breast cancer study and the genetically modified lung cancer study
were captured. In order to justify using a visual based technique such as this, it would
have to be able to show noticeable differences in cell size or shape within a relatively
short amount of time after the cells are obtained. Laser guidance requires little time for analysis and therefore could be applied more quickly if visual comparisons required longer culture periods. In both studies, all cell types had a round morphology immediately after being plated. As seen in figures 6.5 and 6.7, there were no significant differences in cell size in either of the studies. Therefore, in the instances studied here, laser guidance could be used in a timelier manner.

7.3 Basic Cell Sorting

Osteoblasts and fibroblasts were used next to assist in the optimization of the system. Although a low level study compared to others, it was still an important one. The guidance of cells when compared to polystyrene particles is very different. The interaction of the laser with cells, which are nearly transparent in many cases, is much less than that seen with the particles. Therefore, further optimization was necessary with cells to determine optimal laser powers that resulted in ideal guidance events. Fibroblast and osteoblast cells were used for these studies due to the fact that they are fully characterized and familiar cells that are readily available at relatively low cost. From experience obtained during these preliminary studies it was noticed that using lower powers ranging from 50mW to 90mW, the guidance velocity was so slow that the amount of time it took to capture one cell’s profile was unreasonable. However, using much larger laser powers, >200mW, resulted in flirting with the boundary of the cell viability study. Also, with these higher powers came other disadvantages. Most notably was a much greater interaction with the chamber media, PBS. The media began to have an
upward flow that overwhelmed the guidance force. Finally, a sweet spot was found where both cell viability and background forces were unnoticeable. This power was found to be 175mW, at the chamber, and was used for all experiments presented here. Of relatively little importance due to the known resonance effect (fibroblast and osteoblast cells were noticeably different in size), the results did show that these cell types had significantly different velocities.

7.4 Cancer Diagnostics

The ability to diagnose cancer in early stages with high accuracy and confidence is an increasingly interesting topic in today’s society. Using well known models of human breast cancer, generously provided by Dr. Karen Burg’s lab, the laser guidance system was able to not only discern a cancerous cell, 4T1 and 4T07 cells, from healthy breast tissue cells, NMuMG cells, but it also significantly sorted metastatic tumor cells, 4T1, from non-metastatic, 4T07. This was very exciting due to the fact that it is often very difficult to distinguish these cells from one another. After an extensive literature review it was determined that for the cell types used in this study there exist very few ways to determine the potential for metastasis without conducting extensive protein excretion analysis. Although a common trait to distinguish them is a lack of E-cadherin, a surface presenting protein that is important in a cell’s adherence to its surrounding tissue, this form of cancer is unique in that even the cells that do not form tumor nodules at separate regions of the body do still migrate to these locations. Therefore, the assumption that non-metastatic 4T07 cells also lack the E-cadherin marker can be made with confidence.
Ultimately, this makes many of the fluorescence-based techniques ineffective in sorting or diagnosing this form of cancer cell. This study was, therefore, a perfect example of a caser where laser guidance could be effective where other techniques were not. The results of all guidance events were analyzed in two different ways in this study. First, the maximum velocities of the cells were compared, figure 6.15. The results showed that doing so was an effective way to distinguish all three types from each other. Second, the average velocity was calculated for each cell’s total velocity profile. Again, these values were significantly different for all three cell types. Conducting both methods of data analysis was done to see if one was more effective than the other. In this case, either calculated value could have been used. Ultimately, this study was successful proving that laser guidance would be a safe alternative to sorting or diagnosing this particular type of cancer.

7.5 Genetically Modified Cell Sorting

A second study was done to see whether small genetic modifications to a cell would result in large enough variations in the optical properties of the cell that laser guidance would be able to detect. TC-1 cells were genetically modified in a separate collaboration study for the purpose of studying treatment options in lung cancer and were generously provided by Dr. Charlie Wei’s lab. The genetic modification results in a minor adjustment to the cell’s activity as well as the proteins that are synthesized by the cell. It was predicted that these changes in protein content would result in a noticeable change in the refractive index value of the cell as a whole. After analysis using both methods
explained in the cancer cell study above, this was indeed true. Using either the maximum velocity or the average velocity over the entire profile, the TC-1 cells were significantly different from their genetically different sibling, the L-10 cell. This study was of particular interest as the current method of cell sorting used is flow cytometry. However, the fluorescent tags used have to be removed before the cells can be studied. In this removal process, cell viability decreases. Laser guidance would be able to avoid this issue and has the potential to produce larger sample sizes for more effective studies/treatment.

7.6 System Precision

In addition to distinguishing cell types from one another, it was ideal to do so with high repeatability. In other words, it was desirable to have the ability to conduct an experiment and record either the maximum or average velocity of its guidance on one occasion and reproduce those same results on a separate second occasion. From figures 6.18 through 6.25, the results showed that only 3 out of 8 velocity comparisons were effective in producing repeatable results. It was also noticed that the cells used in the genetic modification study had more precise results than in the cancer study. A possible explanation for this may be that the cancer cells have more sensitive characteristics. If so, this could have been a function of variables such as time of day the study was done, cell age, or even environmental changes. It was also possible that the TC-1 and L-10 cells were not as susceptible to these variables. Although this is an issue that should be resolved in future works, it is important to point out that the variation within each cell
type was still not large enough to have an effect on the comparison between two different cell types, which was the ultimate goal of this study.
CHAPTER EIGHT

CONCLUSIONS

The goal of this research was to redesign and further justify and define the sensitivity and possible applications of a laser guidance based method of distinguishing cells. Studies were done to redesign the system for more consistent and reliable experimental conditions, characterize optimal experimental conditions, and conduct specific cell distinguishing experiments.

The resulting experiments produced the following conclusions:

- A redesign in the chamber produced more desirable experimental conditions by reducing the convective forces created by the propagating laser beam.
- Full characterization of optimal system setup including laser power, wavelength, and beam waist size
- Guidance of fully characterized fibroblast and osteoblast cells confirmed that the new setup was able to distinguish phenotypically and morphologically different cell types
- Experimental data shows that it is not possible to distinguish metastatic and non-metastatic breast cancer cells from one another by high magnification microscopy images
- Experimental data shows that it is not possible to distinguish a lung cancer cell from its genetically modified phenotype by high magnification microscopy images
The ability to significantly distinguish healthy breast tissue cells, metastatic breast cancer cells, and non-metastatic breast cancer cells from one another using laser guidance was shown.

The ability to significantly distinguish a murine lung cancer cell from a genetically modified phenotype of the same cell type using laser guidance was shown.

And finally the ability, in many cases, to repeat experiments of a single cell type with no significant discrepancies in their guidance velocities.

These specific examples show the great potential for an alternative method to distinguish phenotypically different cell types from one another based on subtle changes in their molecular content. This novel technique would allow an alternative to currently used techniques without the need for specific cellular markers. Thus, a general live-cell analysis technique allowing detection of small cell-based changes or differences, without additional cell manipulation, has been presented.
CHAPTER NINE

FUTURE WORK

9.1 Further System Optimization

In order to provide more consistent results in the future, the system sensitivity has to be focused on. In order to accomplish this, any sources of variation in the cells need to be identified. For example, it is possible that the cells are expressing different proteins more or less on different days. If this is true then the system will produce non-repeatable guidance velocities and experiments should be conducted on the same day whenever possible.

Cell viability needs to be investigates in this system further. Although previous research in our lab has shown that laser exposure on the scale of this work is not harmful to the cell according to cell outgrowth (with neurons), viability assays, and DNA fragmentation assays, further analysis post-guidance needs to be investigated. For example, cell surface protein expression, growth factor excretion and cell protein excretion analysis are all possible areas of interest to further justify that the system has no effect on the viability of the cells.

9.2 Deflection in a Microfluidics System

Although the chamber was redesigned to minimize the convective forces and create more reliable results, there are still significant limitations. Most notably, the speed at which experiments are conducted is significantly reduced when cells are simply placed in the
chamber and the researcher must wait for a single cell to enter the path of the laser. I have designed two potential solutions to this design flaw, which are illustrated below. Both would incorporate a microfluidics channel (width of an average cell) that cells could be passed through on a single cell level with consistancy, but at a significantly higher throughput, allowing for cells to be analyzed one at a time. A propagating laser traveling against the flow of cells would result in a measurable reduction in speed that would be representative to the cells optical properties, and therefore its phenotype.

Figure 9.1 - Cell sorting based on velocity reduction measurements in a microfluidics system

A second design would incorporate the same microfluidics design but a modified interaction with the laser. A laser propagatinf perpendicular to the path of flow would result in measurable deflections in the cells traveling direction. Again, the magnitude of deflection could be related to the optical properties of the cell and ultimately allowing the experimenter to determine the phenotype of the cell.
9.3 Portable Laser Guidance Microscope

Currently the system is constrained to a large balanced optics table and therefore not
designed to have the ability to be moved from one lab to another. Designing smaller
more portable system would also reduce the amount of space required in a lab as well as
reducing the cost of the product in the future. For this reason, I have designed a portable
laser guidance microscope and begun the preliminary assembly of the system. Detailed
3D solid-edge models have been made and each component is to scale making the
assembly of the system easy for the future. However, the system was not tested or setup
to have the ability to produce guidance events. Future work should intend on including in
it a consideration into the size and portability of the system.
Figure 9.3 – Components and design of a portable laser guidance microscope
Figure 9.4 – External design of a portable laser guidance microscope

Figure 9.5 – Unfinished assembly of a portable laser guidance microscope
APPENDICES
Appendix A

Cell Seeding Protocol

1) Retrieve the cryovial from the liquid Nitrogen tank. Immerse the vial in a water bath maintained at 37 °C and wait till it is fully thawed.

2) Take the cryovial into a clean biohazard hood. Remove the contents of the vial using a pipette and transfer into a centrifuge tube.

3) Rinse the cryovial with 1ml of appropriate media depending on the cell type, pipette up and down and transfer the contents into the same centrifuge tube.

4) Close the centrifuge tube and place in the centrifuge unit. Fill another centrifuge tube with distilled water to the same volume of liquid as the tube containing the cells. Place this in the centrifuge unit at the diametrically opposite location as the tube containing the cells and media.

5) Centrifuge at 1000 RPM for 5 minutes.

6) Check to see the pellet of cells formed at the bottom of the centrifuge tube.

7) Take the tube back into the hood and aspirate the supernatant media. Be careful not to aspirate the cells.

8) Add 3 ml of media into the tube. Break the pellet gently thereby re-suspend the cells in the media.

9) In a T-75 flask, pipette 13 ml of the proper media needed for the cell type.

10) Transfer the contents of the centrifuge with a pipette and into the flask.

11) Label the flask and check under the microscope to see cells floating inside.

12) Spray with 70% ethanol and place in incubator.
Appendix B

Cell Passage Protocol

1) Turn water bath on – push heat button and wait till it heats to about 37 °C
2) Take trypsin from freezer (0.25%) and media from the fridge (media corresponding to the type of cell used) and warm them.
3) Take flask out of the incubator.
4) Tilt it to the side and suck the media out using the vacuum.
5) Put 5 ml trypsin in along the side and shake gently.
6) Set in incubator for about 5 mins.
7) Check under microscope to see if all cells have detached from the bottom of the flask. Else incubate for longer. Also tap flask gently to loosen the cells facilitating easier detachment.
8) Take 3 ml of media and add to the flask.
9) Remove all thus solution using a pipette and put into a centrifuge tube.
10) Take 3 ml more of media, put into flask, shake to collect any cells left at the bottom.
11) Remove the solution and add to the centrifuge tube.[note : total amount of media should be twice as much as the trypsin]
12) Place centrifuge tube in the centrifuge unit. Add a balancing tube having the same volume of liquid as this tube at the diametrically opposite corner. Set centrifuge unit at 1000 rpm for 8 mins.
13) Take tube out and look for cell pellet at the bottom.
14) Suck out the supernatant media.
15) Add PBS to form a dilute suspension of cells in PBS (1000 cells per ml).
Appendix C

Rayleigh Regime

Overview: Consider a paraxial beam of polarized light that illuminates a single spherical particle. If the particle is small compared to the wavelength of the incident light it will experience an instantaneous uniform electric field. The particle will become polarized as the electrons surrounding the atomic nuclei are displaced by an intense electric field. This problem reduces to the known electrostatic problem of an isotropic, dielectric sphere in a uniform electric field. The Cartesian coordinate center is located at the beam waist center, where $z$ is the axial direction of the beam propagation and $x$ is the polarization direction of the electric field.

Equation:

A) Center of particle: 
\[ \vec{r} = (x, y, z) \]
where \( \hat{x}, \hat{y}, \text{and} \ \hat{z} \) are the unit vectors in the $x$, $y$, and $z$ direction

Dipole moment of the particle:
\[ \vec{p}(\vec{r}, t) = 4\pi n_m^2 \varepsilon_0 a^3 \left( \frac{m^2 - 1}{m^2 + 2} \right) \vec{E}(\vec{r}, t) \]

Scattering cross section in the axial direction:
\[ C_{pr,z} = \frac{8}{3} \pi (ka)^4 a^2 \left( \frac{m^2 - 1}{m^2 + 2} \right)^2 \]

Where:
\[ n_m, n_p \] refractive indices of the media and the particle
\[ \varepsilon_0 \] electrical permittivity of free space
\[ m \] relative refractive index given by \( n_p / n_m \)
\[ a \] particle radius
\[ k \] wave number given by \( 2\pi / \lambda \)
\[ \vec{E} \] electric field

B) Forces are a result of two components:

1) The particle, now an oscillating electric dipole, radiates a scattered wave in all directions. The scattered wave changes the direction of the energy flux in the system imparting a momentum change on the particle.
Force due to scattering:

\[
\vec{F}_{\text{scat}}(\vec{r}) = \hat{\cdot} \left( \frac{n_m}{c} \right) C_{pr,z} I(\vec{r})
\]

\[
= \hat{\cdot} \frac{n_m}{c} \frac{8}{3} \pi (ka)^4 a^2 \left( \frac{m^2 - 1}{m^2 + 2} \right)^2 I(\vec{r})
\]

Where:

\( I(r) \) is the incident intensity distribution of the beam

2) The polarized particle experiences the Lorentz force acting on an induced dipole. The time-averaged Lorentz force is the so-called gradient force, which depends on the gradient of the intensity.

Gradient force:

\[
\vec{F}_{\text{grad}}(\vec{r}) = \left( \vec{p}(\vec{r}, t) \cdot \nabla \right) \vec{E}(\vec{r}, t)
\]

\[
= \frac{2\pi n_e^2 a^3}{c} \left( \frac{m^2 - 1}{m^2 + 2} \right) \nabla I(\vec{r})
\]

The above two equations depend on the ability to describe the incident beam intensity. One approach that generates good results when compared with more rigorous methods uses the paraxial beam description[101], a zeroth-order approximation to the Gaussian beam.

Zeroth-order intensity approximation for a Gaussian beam:

\[
I(\vec{r}) = \left( \frac{2P}{\pi \omega_0^2} \right) \frac{1}{1 + \left( \frac{2\hat{\xi}}{1 + \left( \frac{2\hat{\xi}}{\omega_0^2} \right)^2} \right)} \exp \left[ -2(\hat{\xi} + \hat{\eta}^2) \right]
\]

where:

\( \hat{\xi} = x / \omega_0 \)

\( \hat{\eta} = y / \omega_0 \)

\( \hat{\zeta} = z / \omega_0^2 \)

\( \omega_0 = \text{beam waist at the focal point} \)

\( P = \text{Laser Power} \)

Assumes a TEM\(_{00}\) mode at the focus and does not take into account the vector character of the beam[102]. Valid for a weakly convergent beam, but not for a strongly convergent beam.
Appendix D

Ray Optics Regime

Equation:

Force parallel to the direction of each ray:

\[ F_\parallel = \frac{n_m P}{c} \left\{ 1 + R \cos 2\theta - \frac{T^2 \left[ \cos(2\theta - 2r) + R \cos 2\theta \right]}{1 + R^2 + 2R \cos 2r} \right\} \]

And orthogonal to the direction of each ray:

\[ F_\perp = \frac{n_m P}{c} \left\{ R \sin 2\theta - \frac{T^2 \left[ \sin(2\theta - 2r) + R \sin 2\theta \right]}{1 + R^2 + 2R \cos 2r} \right\} \]

Where:

- \( R \) Fresnel Reflections coefficient
- \( T \) Fresnel transmission coefficient
- \( \theta \) angle of incident rays
- \( r \) angle of refracted rays

Forces are affected by the polarization of the beam, since \( R \) and \( T \) depend on the polarization of the rays relative to the plane of incidence. The forces are summed over all rays and give a good estimate of the scattering phenomenon. However, scattering is only half the problem since, according to van de Hulst, “the diffraction pattern formed behind the sphere is the very narrow, very intense and concentrated near the forward direction; It arises from the incompleteness of the wave front passing the sphere”[80], that is, from rays missing the sphere. The radiation contained in both the reflection-refraction pattern and the diffraction pattern equals the total energy incident on the particle.
Appendix E

Generalized Lorentz-Mie Regime

Optical Force Generated by a Weekly Focused Laser Beam on Dielectric Spheres

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Introduction

The study of interaction of light with tissue is very important and significant. Optically tissue can be characterized by studying the light propagation in it. The physiological condition or state of single cells or tissues is expressed through (but not exclusively) changes in cell size or refractive index. Changes in refractive index or cell size influence the optical properties. Measuring or analyzing the light scattering may thus provide information about the cell(s) or tissue.

The tissue can be ideally studied for its scattering, refractive index, and absorption properties. The scattering arises from: cell membranes, cell nuclei, capillary walls, hair follicles etc. The absorption arises from: hemoglobin and melanin and also water and the existence of molecular vibration/rotational states.

Interaction between a light and the particle

When a light wave is incident on a particle then the wave interacts with the particle in various ways. To study this the wave can be modeled as an oscillating electrical field vector. Now this field induces dipoles in the particle. An oscillating dipole emits radiation in all directions which is a direct property from the Classical Electromagnetic theory.

More specifically we are interested in the Elastic Light Scattering. By elastic scattering we mean that the frequency of emission of the radiation has the same frequency as the induced oscillating dipoles, which is again the same as that of the incident wave.

Parameters which influence this interaction

Now that we know the interaction of light and particle can be used to study it, we need to develop quantified methods to do the same. The Scattered light intensity can be written as a function of

1) Wavelength of incident radiation
2) Refractive index of the particle (Higher the refractive index of particle relative to medium; the more the light scattered )
3) Size of the particle
4) Angle made with respect to the incident beam

Hence to solve these mathematically we have the various theories to study the same. For small particles whose size is much lesser than the wavelength of the incident light we have the Rayleigh theory. For very large particles the main Geometric theory or the franhauber theory is used. Now for the intermediate size particles we have the famous Mie theory.

Most of our biological samples lie in this region and hence study of the Mie theory is a major area of interest.

**Laser beam and optical forces associated with it**

A laser beam is a coherent electromagnetic source of light which is highly focused and directional in nature. When a laser beam is incident on a particle then the particle experiences two kinds of forces called the
1) Gradient force
2) Scattering force

The gradient force is the one which pushes the particle towards the centre of the beam which is the region of maximum intensity of the beam.

The scattering force is the resultant of all forces due to the radiation pressure on the particle. Incident radiation can be absorbed and isotropically reemitted by atoms or molecules. With this, two impulses are received by the molecule, one along the beam propagation of the incident light and one opposite to the direction of the emitted photon. Since the photon emission has no preferred direction, a net force results in the direction of incident photon flux. This force is directed along the propagation of light.

**Mie Theory**

Now to quantitatively study this phenomenon of scattering we need to use the mie theory to evaluate the parameters. The Mie theory is a complete mathematical-physical theory of the scattering of electromagnetic radiation by spherical particles, developed by Gustav Mie in 1908.

In this we assume that the Mie theory embraces all possible ratios of diameter to wavelength of the particle to the incident beam. It also assumes a homogeneous, isotropic and optically linear material irradiated by an infinitely extending plane wave.

According to the Mie theory, the scattering angular pattern is symmetrical along the axis of incident light for perfect spheres. That is, the light scattering pattern is the same for the same absolute value of the scattering angle. Also, the light intensity is higher
for larger sphere at the same scattering angle. This implies that large particles can be
distinguished from small particles by the strength of light reflected off their surfaces at
the same angle. In addition, the light intensity gets lower as the scattering angle increases.
However, this trend of angular dependence of light intensity is reduced when the particles
are very small (in the nm range). The scattered angular light intensities are
indistinguishable from each other when the particles are smaller than 50 nm. Another
feature for light scattering of a sphere is that there are maxima and minima for the
angular light intensity observed. The pattern is characteristic for a particle of a given size.

Although the angular light intensity (flux) distribution (pattern) is a complicated
function of many variables, most are known constants in a direct instrumental
measurement. Thus, as long as the light flux distribution is obtained, the particle size
(diameter) can be worked out.

Scattering forces in GLMT are given by:

\[
\vec{F}(\vec{r}) = \left( \frac{n_m}{c} \right) \frac{2P}{\pi \omega_0^2} \left[ \hat{x} C_{pr,x} (\vec{r}) + \hat{y} C_{pr,y} (\vec{r}) + \hat{z} C_{pr,z} (\vec{r}) \right]
\]

(3.1)

Where:

\[ C_{pr,x}, C_{pr,y}, and C_{pr,z} \] are the cross sections for radiation pressure as
presented in the original work of Gouesbet et al.[86].
Appendix F

$M^2$ Calculations

The following ratio provides a dimensionless parameter that gives an accurate indication of the propagation characteristics of the beam.

$$M^2 = \frac{w_{0R} \cdot \theta_R}{w_0 \cdot \theta}$$

where:

- $w_{0R} =$ beam waist of the real beam
- $\theta_R =$ far-field divergence of the real beam

With $M^2$ defined, it is now possible to more accurately determine the propagation characteristics of a real beam, as well as apply it to modify the lens equation. A purely Gaussian beam, $M^2 = 1$, the beam-waist beam-divergence product is given as:

$$w_0 \theta = \frac{\lambda}{\pi}$$

Therefore, for a real laser beam, we have

$$w_{0R} \theta_R = \frac{M^2 \lambda}{\pi} > \frac{\lambda}{\pi}$$

where:

- $w_{0R} =$ 1/2 int ensity waist radius
- $\theta_R =$ 1/2 int ensity far-field divergence angle

Finally, we can plug these values into the propagation equations.

$$w_R(z) = w_{0R} \left[ 1 + \left( \frac{z \lambda M^2}{\pi w_{0R}^2} \right)^2 \right]^{1/2}$$

and

$$R_R(z) = z \left[ 1 + \left( \frac{\pi w_{0R}^2}{z \lambda M^2} \right)^2 \right]$$

where

- $w_R(z) =$ 1/e^2 int ensity radius of the beam
- $R_R(z) =$ 1/e^2 int ensity beam wavefront radius at $z$

The definition for the Rayleigh range of a real laser beam remains the same.
Finally, when it is allowable to assume a truly Gaussian profile, $M^2=1$, the equations reduce to

\[ R(z) = z \left[ 1 + \left( \frac{\pi w_0^2}{\lambda z} \right)^2 \right] \]

and

\[ w(z) = w_0 \left[ 1 + \left( \frac{\lambda z}{\pi w_0} \right)^2 \right]^{1/2} \]

In order to find the beam waist radius that minimizes the beam radius at distance $z$ the following equation is used

\[ w_0(\text{optimum}) = \left( \frac{\lambda z M^2}{\pi} \right) \]

Finally,

\[ z_R = \frac{\pi w_0^2}{\lambda} \]

where

\[ z_R = \text{Raleigh range} \]

In this application it is necessary to focus, modify, and shape the laser beam utilizing a variety of precisely placed lenses and optical elements. The $M^2$ value can be utilized to correct for a real beam when using the lens equation as well.

\[ \frac{1}{s + (z_R / M^2)^2 / (s - f)} + \frac{1}{s''} = \frac{1}{f'} \]

and the normalized equation transforms to

\[ \frac{1}{(s / f) + (z_R / M^2 f)^2 / (s / f - 1)} + \frac{1}{(s'' / f)} = 1 \]
Appendix G

Waist Size Calculations

The method and the formulas used are as follows:-

We know that the intensity distribution of a Gaussian beam is given by,

1. \[ I(x, y) = \frac{2P_0}{\pi \omega^2} \exp\left(-\frac{2x^2 + 2y^2}{\omega^2}\right) \]

Where
- \( P_0 \) = total laser power
- \( x, y \) = coordinates of the plane vertical to the beam axis (origin is the centre of the beam)
- \( \omega \) = \(1/e^2\) beam radius

When the knife cuts into the beam vertically and the transmitted power will be related to

2. \[ P(x) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} \frac{2P_0}{\pi \omega^2} \exp\left(-\frac{2x^2 + 2y^2}{\omega^2}\right) \partial x \partial y \]

Eqtn 2 can be separated using separation of integrals as

3. \[ P(x) = \int_{-\infty}^{\infty} e^{-\frac{2x^2}{\omega^2}} \partial x \int_{-\infty}^{\infty} e^{-\frac{2y^2}{\omega^2}} \partial y \]

Since the two integrals are similar, we can solve one integral using the following substitution and use the same method for the other integral. So using the substitution that

\[ t = \frac{\sqrt{2}x}{\omega} \]

We can rewrite one integral of Eqtn 3 as

4. \[ \frac{\omega}{\sqrt{2}} \int_{-\infty}^{\infty} e^{-t^2} dt \]

Now from integration formulas, an error function is defined as (as in mathematica)
\[ erf(x) = \frac{2}{\sqrt{\pi}} \int_{0}^{x} e^{-t^2} dt \]

5. \[ erf(\infty) = 1 \]
\[ erf(-\infty) = -1 \]

Using 4 and 5 we get

\[ \int_{-\infty}^{\infty} e^{-t^2} dt = \frac{\omega}{\sqrt{2}} \sqrt{\pi} \]

Substituting 6 in 3 we get

\[ P(x) = \int_{x}^{\infty} P_0 \sqrt{\frac{2}{\pi \omega}} \frac{1}{\omega} \exp\left(-\frac{2x^2}{\omega^2}\right) dx \]

**To solve for matlab we do the following**

Assume \( t = \frac{\sqrt{2}(x - \mu)}{\omega} \)

Then differentiating both sides, we get

8. \[ dt = \frac{\sqrt{2} d(x - \mu)}{\omega} \]

Thus using 8, we can write equation 7 in terms of t such that

9. \[ P(x) = \frac{P_0}{\sqrt{\pi}} \int_{\infty}^{\infty} \exp(-t^2) dt \]

We express this final Eqtn 9 in terms of the error function defined in 5 as
This is the equation we use to curve fit in matlab.

Now the second step is to we repeat the procedure moving the knife along the Z-axis and taking the same set of readings again for every Z we get

a. \[ W(z) = W_0[1 + \left( \frac{Z - Z_0}{Z_0} \right)^2]^{\frac{1}{2}} \]

Where \( W(z) \) is the width of the beam.

b. \[ W(z) = \sqrt{W_0^2 + W_0^2 \left( \frac{Z - Z_0}{Z_0} \right)^2} \]

We know that

c. \[ W_0 = \left( \frac{\lambda Z_0}{\pi} \right)^{\frac{1}{2}} \]

Substituting c in b we get

\[ W(z) = \sqrt{W_0^2 + W_0^2 \left( \frac{Z - Z_0}{Z_0} \right)^2} \]

\[ W(z) = \sqrt{W_0^2 + \left( \frac{\lambda Z_0}{\pi} \right) \left( \frac{Z - Z_0}{Z_0} \right)^2} \]

Multiplying and diving by \( \frac{\lambda}{\pi} \)
Again above is the equation we use to curve fit in matlab.

From the above we can substitute the value of Z and get \( W(z) \) from which we can obtain the waist radius since the \( W(z) \) assumes the minimum value at \( W_0 \) in the plane \( Z = 0 \).
REFERENCES


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