The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, a thesis/dissertation entitled:

Selective Laser Arrest of Antibody-Producing Cells to Increase Antibody Production

submitted by Jeffrey Chiu in partial fulfillment of the requirements for the degree of Master of Applied Science in Mechanical Engineering

Examining Committee:
Hongshen Ma, Mechanical Engineering Supervisor
Ryozo Nagamune, Mechanical Engineering Supervisory Committee Member
Boris Stoeber, Mechanical Engineering Supervisory Committee Member

Additional Examiner

Additional Supervisory Committee Members:
Abstract

Monoclonal antibodies have become a dominant biopharmaceutical in recent years, with sales expected to exceed $125 billion by 2020. Antibody therapies have been used to treat a wide range of diseases including cancer, multiple sclerosis, and rheumatoid arthritis, with higher specificity and lower toxicity than other chemotherapeutics. The potential promise of antibody therapies has necessitated a significant need to develop improved production technologies in order to shorten the timelines for development, testing, and clinical trials.

Modern methods of monoclonal antibody production involve transfecting an antibody gene expression cassette into a host cell line for production, where the cassette is randomly integrated into the genome. This random integration results in a heterogeneity between transduced cells, resulting in significant variability in antibody production rate within the cell population. Additional screening and selection processes are therefore needed to optimize the productivity of the antibody-producing cell line. While several strategies have been developed to select high-producing cell lines, each existing strategy suffers from problems such as long timelines, indirect selectivity, complex procedures, and proprietary processes.

We developed a technology named Selective Laser Gelation (SLG) capable of selectively arresting the growth of individual target cells. This capability is enabled by localized gelation using an infrared laser to utilize the unique inverse solution-gel transition of methylcellulose solutions. Phase-transition hysteresis enables the retention of localized gels after the laser is removed. Methylcellulose solution limits the diffusion of secreted antibody from individual cells and small colonies, and when combined with a fluorescently-conjugated secondary antibody, the produced antibody can be visualized and quantified. This capability is then used to selectively preserve high antibody-producing cells while arresting the growth of low-producing cells. In this thesis, we first modeled the thermodynamics of laser heating on a methylcellulose solution. We then developed an experimental apparatus and software to test the SLG procedure, which we used to show that the SLG process can selectively inhibit the growth of selected cells. Finally, we use the SLG process to increase overall antibody productivity within a shorter timeline than current methods by selecting high-producing antibody-secreting cells.
Lay Summary

Monoclonal antibodies are a type of molecule produced by certain types of cells that can be used as medicine to treat many diseases, such as cancer, multiple sclerosis, and rheumatoid arthritis. The process for developing the cell lines that produce monoclonal antibodies can create dramatic differences between the amounts of antibodies produced by each cell. In order to maximize the overall amount of antibody produced, we developed a method to selectively stop the growth of cells that produce low amounts of antibody. We achieve this property using the unique physics of a material that can change from liquid to solid after being heated using a laser spot. We show that by stopping the growth of low-producing cells, we can dramatically increase the overall production of the producing cell line.
Preface

The entirety of this thesis including process design, system design, software, and biological experiments were all performed by the author.

A version of this thesis is being prepared for publication with the assistance of Dr. Hongshen Ma, Dr. Kerryn Matthews, and Dr. Simon Duffy.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>DHFR</td>
<td>Dihydrofolate Reductase</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Array</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GOI</td>
<td>Gene of Interest</td>
</tr>
<tr>
<td>GS</td>
<td>Glutamine Synthetase</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IMDM</td>
<td>Isocove’s Modified Dulbecco’s Medium</td>
</tr>
<tr>
<td>LEAP</td>
<td>Laser Enabled Analysis and Processing</td>
</tr>
<tr>
<td>MC</td>
<td>Methylcellulose</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MSX</td>
<td>Methionine Sulfoximine</td>
</tr>
<tr>
<td>MTX</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PEGDA</td>
<td>Poly(ethylene glycol) Diacrylate</td>
</tr>
<tr>
<td>PWM</td>
<td>Pulse Width Modulation</td>
</tr>
<tr>
<td>SLG</td>
<td>Selective Laser Gelation</td>
</tr>
<tr>
<td>UI</td>
<td>User Interface</td>
</tr>
<tr>
<td>WPF</td>
<td>Windows Presentation Foundation</td>
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My parents Priscilla and Billy, and my sister Jacqueline for their unwavering support

Joan for believing in me

Thank you
Dedication

To my mom Priscilla and my dad Billy. Thank you for everything.
Chapter 1: Introduction

Antibodies are proteins naturally produced by B-lymphocytes in the immune system to identify and neutralize foreign molecules [1]. Currently, the use of monoclonal antibodies (identical antibodies produced by genetically identical cells) have shifted from their use in the research and diagnosis to fast becoming a dominant therapeutic, and may soon account for the majority of the biopharmaceutical market [2]. Their increased specificity and decreased toxicity [3], [4] have given monoclonal antibody therapies significant advantages over traditional chemotherapeutics. These advantages have accelerated the demand for new monoclonal antibody therapies. In order to meet the demand for monoclonal antibody research, clinical trials, and commercialization, improved production methods to increase product yield and decrease timelines are required.

The history of monoclonal antibodies begins in 1975, where Khöler and Milstein developed the method to produce murine monoclonal antibodies in vitro using hybridoma [5]. Before this discovery, it was not possible to produce monoclonal antibodies at significant scale, and for the significance of their work they received the Nobel Prize in Medicine or Physiology in 1984 [6]. Since then, monoclonal antibodies are the fastest growing category of human therapeutics and the second largest group of therapeutics after vaccines [7]. They are used to treat diseases in humans such as cancer, multiple sclerosis, and rheumatoid arthritis among others. The ability of monoclonal antibodies to bind to a target with high specificity and high affinity, and the ability to create humanized sequences to a target have created a large demand for these products. The first monoclonal antibody was approved and commercialized in 1986, and as of 2014, 44 monoclonal antibodies are approved in Europe and the United States. In 2013, sales neared $75 billion, almost half of the total sales of all biopharmaceutical products and sales of monoclonal antibodies are expected to exceed $125 billion by 2020 [2].

The process to achieve a stable cell line capable of producing the desired antibody is long and complex. In general, mice are immunized with the target antigen and B-lymphocytes are obtained from the spleen several weeks later. B-lymphocytes are then fused with immortal myeloma cells to create hybridoma. After fusion, hybridoma cells are plated in selective media in multi-well plates. The plates are then screened for the antibody specific to the desired target.
antigen. Hybridoma in the positive wells are then expanded and cloned, resulting in an immortal cell line capable of producing a specific antibody [8].

Instead of using hybridoma as the cell line for monoclonal antibody production, modern methods of antibody production sequence the antibody encoding gene of interest (GOI) from the hybridoma. The expression vector including the GOI is then transfected into a host cell such as a Chinese Hamster Ovary (CHO) or Human Embryonic Kidney (HEK) cells. CHO cells are the most popular host, one reason being that they have been proven to be safe for over two decades, making it easier to obtain regulatory approval [9]. Figure 1-1 shows the typical process from transfection of the expression vector into the host cell to the expansion of selected cells for further evaluation.

![Diagram of antibody production process](image)

Figure 1-1: The typical process from transfecting the expression vector including the gene of interest (GOI) to expansion for further evaluation. Adapted from Lai, T. et al. [10] under CC-BY-3.0.
Post transfection, the product yield must be maximized in order to meet the demand of antibody that is required for clinical trials and commercialization. The transfected clones are screened and selected, then adapted for suspension and serum-free culture conditions which improves batch-to-batch consistency. Clones are then scaled up to industrial sized bioreactors and cultured for an extended period of time, after which the produced antibodies are collected and purified. Cell selection, culture adaptation and optimization, and scale-up processes can all affect the final yield of the cells [11].

In order to maximize the monoclonal antibody produced by the transfected cells, many methods of screening and selection currently exist to improve the cell line productivity. The antibody production capabilities of the cells are measured, and then the cells with high specific production are selected to be cultured. Selecting cells with high specific productivity results in a higher yield of antibody per cell as the cell culture grows compared to beginning the cell culture with a mixture of high-producing and low-producing cells [12].

1.1 Selection of High-Producing Clones

The transfection of the gene of interest (GOI) into host CHO cells introduces significant heterogeneity between cells, which is caused by the random integration of the GOI into the host genome [10], [13]. Heterogeneity within the cell population causes variances in growth rates and specific antibody productivity within the cell population [14], [15] that can lead to the cells producing high amounts of antibody only making up a small percentage of the total population [16]. In addition, high-producing cells use more metabolic energy for antibody production; whereas low and non-producing cells reserve more energy towards growth [12]. As a result of the small proportion of high-producing cells and the metabolic energy differences, the low producing population will outgrow the high-producing population over time. For example, a growth rate advantage of 9% is sufficient for low-producing cells to dominate the culture after 25 passages [17], resulting in reduced overall antibody yield to decrease. Due to the differences in growth rate, it is important to identify and isolate the high-producing cells that make up a small percentage of the overall population prior to scale-up in order to maximize antibody production.
Background - Current Methods for Selection of High-Producing Cells

Many methods have been developed for the selection of high antibody-producing cells. The methods include limiting dilution, gene amplification, fluorescence activated cell sorting, semi-solid cloning, and laser-enabled analysis and processing.

1.1.1 Limiting Dilution

Limiting dilution selects high-producing cells by diluting the cell suspension to a very low density and then seeding the suspension into a standard multi-well plate for further cell culture (Figure 1-2) [18], [19]. The objective is to statistically deposit single cells into single wells in a multi-well plate, such that the duplication of single cells in a monoclonal population. For example, cells are diluted to approximately 8 cells/ml and then 100 µl of the diluted solution are plated into each well of a 96-well plate, resulting in an average of 0.8 cells per well. However, each well may receive zero, one, or more than one cell. The cell distribution is modeled as a Poisson distribution (Equation (1.1)) where $k$ is the number of cells in each well and $\lambda$ is the average number of cells per well.

$$P(k) = e^{-\lambda} \frac{\lambda^k}{k!}$$  (1.1)

Figure 1-3 shows the probability of having 0, 1, 2…6 cells per well with different average cell densities ($\lambda$). Wells that have more than one cell $P(k > 1)$ are undesirable, as the expanded
population will no longer be monoclonal, enabling low antibody-producing cells to outgrow within the population. The more diluted the cell sample is, the higher probability of having no more than one cell per well $P(k \leq 1)$. However, a more diluted sample also increases the number of empty wells $P(k = 0)$ and a key limitation is that single cells must be cultured for a period of days or weeks before well occupancy can be confirmed, resulting in long timelines [12]. This culture period is also required for quantification of antibody secretion, which is normally determined by Enzyme-Linked Immunosorbent Assay (ELISA). Again, the need to culture the cells for an extended period of time becomes a liability, as fast growing cells may dominate the culture and mask high-producing clones. This distinction between fast-growing and high-producing clones may not become apparent until the culture is expanded to production-scale. Furthermore, ELISA is performed on the entire multi-well plate regardless of whether there is cell outgrowth, making empty wells both laborious and an economic waste. Despite these drawbacks, limiting dilution is simple to perform and relatively inexpensive compared to methods that require specialized equipment, thus is still widely used in both research and industry.

Figure 1-3 The probability of a certain number of cells per well $P(k)$ follows a Poisson distribution and varies depending on the average number of cells per well ($\lambda$). A lower average number of cells per well decreases the chance of having more than one cell per well but also increases the number of empty wells.
1.1.2 Gene Amplification

Gene amplification methods to select high-producing cell lines require the use of a selective marker such as glutamine synthetase (GS) or dihydrofolate reductase (DHFR) to be transfected along with the GOI. The presence of the selective marker is then amplified by only selecting for the cells which contain high copy numbers and expression of the selective marker (Figure 1-4) [20]. The transfected cells express different levels of the selective marker due to random genomic integration of the transfection process. Cells that express lower levels of the selective marker are inhibited from growing by adding a specific drug.

When DHFR is used as the selective marker, after transfecting the combined DHFR and GOI into the host cell, the drug methotrexate (MTX) is added to culture media at increasing concentrations. MTX inhibits cells without DHFR from growing, and the increasing concentrations of MTX selects only cells which have amplified amounts of DHFR. Cells with amplified DHFR also have amplified GOI, which correlates to higher protein production levels [14]. Similarly, the GS system adds methionine sulfoximine (MSX) to the culture media to suppress cells without GS. The result is only cells transfected with GS and the GOI survive.

Gene amplification systems offer higher throughput than limiting dilution. However, there are many drawbacks. The time required to develop an acceptable cell line is typically very long, up to six months to produce viable candidates. The selection method only indirectly selects for the marker, and not directly the GOI. This can result in cells that have high expression of the selection marker but have lost the GOI. Additionally, there can be instability with the amplified
genes [20]. The result of all of these drawbacks is that even after the gene amplification process, there is no guarantee that the resultant cells have a higher specific antibody productivity [15]. Cells selected using gene amplification will likely have to go through another cell selection method to select the high-producers in the remaining population.
1.1.3 **Fluorescence Activated Cell Sorting (FACS)**

Fluorescence activated cell sorting (FACS) to select high-producing cells involves suspending the cells in a fluid and rapidly passing the fluid through a narrow channel where a laser and optical detectors are used to detect antibody production based on fluorescence signal (Figure 1-5) [18]. To measure the antibody production level of individual cells, the antibody production must be converted into a fluorescence signal. After the measurement of antibody production, cells can then be sorted into different collection tubes or individual wells using an electrically charged deflector plate.

![Image of FACS principle](image)

Figure 1-5 Working principle of fluorescence activated cell sorting (FACS), adapted from Adapted from Gross et al. [18] under CC-BY-4.0.

Standard FACS systems can detect cell-associated antibody bound to the cell surface, but cannot detect antibodies secreted from the cell [20]. Measurement of secreted antibody using FACS is possible using gel microdrop secretion assay (Figure 1-6) [12], [21]. Cells are encapsulated in a biotinylated agarose droplet, and secreted antibodies are detected using a fluorescently-conjugated secondary antibody or specific antigen. The fluorescence intensity can then be
measured using FACS and correlated to the amount of secreted antibody [10]. The disadvantage of this process to measure secreted antibodies using FACS is that it is technically complex and time consuming [12].

The advantages of using FACS to select high-producing cells include high throughput and the ability to isolate cell populations even at a low frequency. Some of the disadvantages of FACS are cell death caused by shear forces, low recovery rate requiring a large cell sample (exceeding 10,000 cells), and the need for pre-treatment of the cell sample [22].

![Figure 1-6 Gel microdrop secretion assay for detection of secreted antibodies [21]. Reprinted by permission from Springer Nature: Nature Medicine. Gel microdrop technology for rapid isolation of rare and high producer cells by Weaver, J et al. © Springer Nature 1997.](image)

### 1.1.4 Semi-solid Cloning

Instead of growing cells in standard liquid media, cell colonies can be isolated by placing them in semi-solid media [23]. Plating cells in semi-solid media such as agar, agarose, or methylcellulose allows individual cells to grow into distinct colonies (Figure 1-7) that can be manually picked using a micromanipulator or pipettor, or automatically picked using an automated colony picker. Manual methods have the advantage of being relatively inexpensive compared to automated systems. However, both manual picking with a micromanipulator or pipettor are low-throughput. A micromanipulator allows for earlier picking of the cell colonies, but is very labor intensive [24] whereas picking cells with a pipettor is much easier but the cell colonies must grow large enough to be easily seen (Figure 1-8) [25].
Figure 1-7 Single CHO cells plated in methylcellulose media grow into distinct colonies.

Figure 1-8 Hybridoma cell colonies after 14 days grown in methylcellulose-based semi-solid media, ready to be hand-picked using a 10uL pipettor [25]. Reprinted by permission from Springer Customer Service Centre GmbH: Springer. Simultaneous Cloning and Selection of Hybridomas and Transfected Cell Lines in Semisolid Media by Wognam, B and Lee, T. © Springer Science+Business Media, LLC 2013.
In addition to single cells growing into discrete colonies, semi-solid media also limits the diffusion of secreted proteins. Caron et al. separated high-producing cells secreting recombinant protein IGF-E5 by plating the cells in methylcellulose-based media (Figure 1-9) [26]. This method is marker-free and only requires a fluorescent secondary antibody against the secreted protein. The semi-solid cloning method measures secreted proteins over the time interval they are cultured, whereas FACS methods indirectly measure the secretion at the time of analysis [20].

Semi-solid cloning can be a high-throughput method when using an automated colony picker. Figure 1-10 shows one model of an automated colony picker. A camera takes a picture of the petri dish and software algorithms determine which colonies are to be picked based on factors such as colony size, roundness, and protein secretion. A robotic arm uses a new sterile pipette tip to pick each colony and transfer it into a new plate. Not only does the use of an automated colony picker improve precision of desirable colony selection, it also achieves high throughput at the time of picking (imaging and picking 10,000 cell clones in 1 hour [10]). However, even automated colony pickers require that colonies be grown for up to seven days before they can reliably detect and extract the colonies. [12].
1.1.5 Laser-Enabled Analysis and Processing (LEAP)

Laser-Enabled Analysis and Processing (LEAP) is a proprietary negative selection process developed by Cyntellect that extends the advantages of clone selection in a microtiter plate. Like semi-solid media, the LEAP process limits the diffusion of secreted antibody but instead does so by incubating cells in wells which contain a protein G capture matrix. The protein G is then blocked to allow a fluorescently-labeled secondary antibody to detect the secreted antibody. Unlike other automated systems that use disposable needles and a robotic arm to isolate desirable cell colonies, the LEAP mechanism uses a laser for photothermal, photochemical, or photomechanical cell purification [27]. Cell selection is aided by software which ranks each cell based on secreted antibody in the localized area around the cell as well as other factors such as area and proximity to other cells. The highest ranked cell in each well is retained while a laser eliminates the other cells by photomechanical cell lysis. In contrast to visual colony picking that requires several days of culture, the LEAP system requires 6-48 hr of incubation in the microtiter plate, cell staining, and less than 30 seconds for the imaging laser to select high-producing cells.
from a population of 10,000 cells. This process increases antibody secretion of a cell population 5 to 20-fold [28]. Furthermore, since LEAP does not require disposable needles that make standard automated systems costly and prone to sample contamination.

While the LEAP system is high throughput and can detect secreted proteins, it is a proprietary process, requires specially treated microtiter plates, and multiple staining and washing steps are required. Additionally, it is possible for the laser to damage the cell that is to be preserved in the well [12].

1.1.6 Comparison of Selection Methods

An overview of the advantages and disadvantages of the methods used to select high-producing clones described in section 1.1.1 to 1.1.5 are presented below in Table 1-1. While many of the methods can be automated using robotics for higher throughput, there are innate advantages and disadvantages to each method. Chapter 2 and onwards describe the development of a novel cell selection process that allows for simple detection of secreted antibody level, while having shorter timelines than traditional cell line development.

Table 1-1 Comparison of common selection methods for high-producing antibody cells

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
</table>
| Limiting Dilution | • Simple  
• No equipment required | • Long timeline  
• Low throughput |
| Gene Amplification | • No equipment required  
• Higher throughput than limiting dilution | • Long timeline  
• Indirect selection |
| FACS        | • High throughput | • Secreted antibody measurement requires complex gel microdrop encapsulation |
| Semi-Solid  | • Secreted antibody is diffusion limited  
• Cells are grown into separate colonies | • Colonies must be grown for up to two weeks before picking |
| LEAP        | • High throughput  
• Can measure secreted antibody | • Requires several staining and washing steps  
• Laser can damage inadvertent cells  
• Proprietary process |
1.2 Research Goals

This thesis describes a new technology that uses selective laser gelation (SLG) of methylcellulose to selectively enable the growth of high-producing cells in order to develop high-producing cell lines for antibody production. SLG restricts the growth of cells by using a laser to locally form a hydrogel that encapsulate and arrest the growth of target cells. The gel is retained due to gelation hysteresis after the laser is removed. This approach is equally effective for selecting both suspension and adherent cell lines in standard microtiter plates, and the procedure retains a non-contact sterile environment. We demonstrate that this approach enables the selective growth of high-producing CHO cell colonies from a mixed input population, which facilitates antibody production in a shorter timeline and in manner that is amenable to automation. The goals of the thesis are as follows:

1. Investigate the material properties of methylcellulose in order to develop a model of localized thermal gelation
2. Develop an experimental apparatus capable of testing localized laser gelation
3. Confirm selective cell growth using the laser gelation process using live cells in vitro
4. Develop both hardware and software pipeline to select high-producing antibody-secreting cells
5. Show that selective laser gelation can improve the overall yield of antibody production

Chapter 2 of this thesis describes the material properties of methylcellulose, a heat transfer model of the localized thermal gelation, and simulations of the gelation area with varying laser characteristics. In Chapter 3, an experimental apparatus is constructed and used to validate the gelation simulations, the software and automation of the system is described, and the SLG process of inhibiting cell growth on live cells in vitro is validated. Chapter 4 lists the materials and methods required for experimental testing of the SLG process. The results of the experiments are described and discussed in Chapter 5. Chapter 6 includes the conclusion, limitations, and future work of the project.
Chapter 2: Design and Modeling

2.1 Selective Laser Gelation Overview

Selective Laser Gelation (SLG) leverages a unique property of methylcellulose solutions, which exist in liquid form at low temperatures and solidifies to a hydrogel when temperature is increased [29]. In addition, methylcellulose solutions exhibit phase-transition hysteresis, where the temperature required to transform from solution to hydrogel is greater than the temperature required to transform hydrogel to solution [30]. These effects combine to make it possible to selectively transform a small region of methylcellulose from solution to gel using localized laser heating, and for the gelled area to be retained after removing the laser. Since methylcellulose solution is also a common cell culture media, cells can be grown in methylcellulose solution and regions containing certain cells can be selectively solidified into a gel to selectively inhibit growth (Figure 2-1). By culturing antibody-producing cells in methylcellulose solution, secreted antibodies remain in the vicinity of the cell that produced them, allowing for earlier detection in the order of a couple of days. Laser gelation provides a non-contact selection method that reduces the risk of contamination and is more amenable to automation.

![Figure 2-1 Overview of the Selective Laser Gelation (SLG) process. Cells are mixed with methylcellulose (MC) solution. A laser is applied through a focusing objective towards a target cell, causing the MC to undergo gelation in the region around undesired cells. The gel region prevents the undesired cells from growing.](image-url)
In order to create an experimental apparatus is to test the SLG method on cells *in vitro*, several factors influencing the design of a system capable of creating a small localized gelation area in methylcellulose solution are described in this chapter. The material properties of methylcellulose relating to thermal gelation are first studied. A heat transfer model is proposed based on laser absorption and heat conduction of methylcellulose. A finite element model of the system was created to simulate the temperature distribution in order to determine the gelation area. The remainder of Chapter 2 describes the design and simulation of the SLG system.
2.2 Methylcellulose Material Properties

Methylcellulose is typically added to the culturing media of antibody producing cell to provide a semi-solid matrix to immobilize cells and enable fluorescence detection of secreted antibodies in order to enable manual picking of desirable colonies [23], [25]. Methylcellulose solution undergoes a reversible inverse solution-gel phase transition upon heating [31], [32]. Unlike most other hydrogels, methylcellulose solutions solidify into a gel upon heating rather than cooling [29]. The phase transition also exhibits a hysteresis behavior where the transition temperature from solution to gel upon heating is a higher temperature than the temperature which the gel dissolves into a solution during cooling ($T_{gel} > T_{dis}$) [30]. The storage modulus ($G'$) as a function temperature of a typical methylcellulose solution (Figure 2-2) shows the inverse solution-gel phase transition property and hysteresis effect.

![Figure 2-2 Gelation curve of a 1.5% methylcellulose (Dow Chemical Company METHOCEL™ A4M) solution. The storage modulus ($G'$) is plotted on the y-axis. Red arrow indicates $G'$ during heating, blue arrow indicates $G'$ during cooling. Optical micrograph pictures are shown at different points of the heating and cooling cycle [30]. Reprinted by permission from American Chemical Society: Langmuir. Interplay between Gelation and Phase Separation in Aqueous Solutions of Methylcellulose and Hydroxypropylmethylcellulose by Fairclough, J et al. © American Chemical Society 2012.](image)

The transition temperature from solution to gel and vice versa is dependent on the concentration of the dissolved methylcellulose [33]. Controlling the concentration of methylcellulose to
approximately 1-2% results in a semi-solid solution that will have $T_{gel} > 37^\circ C$ and $T_{dis} < 37^\circ C$. The temperature $37^\circ C$ is important as it is the temperature at which cell culture typically takes place inside an incubator. By controlling the reversible gelation around $37^\circ C$, the methylcellulose solution will be at the solution phase prior to the SLG process, and remain in gel phase even after the laser is removed from the target cell and the entire cell/methylcellulose mixture is incubated for cell culture.

The inverse solution-gel transition of methylcellulose solution can be seen in Figure 2-3. A vial containing 1 mL of 2% methylcellulose solution was heated from $20^\circ C$ to $60^\circ C$ in an oven with the vial upright. The vial then tipped on its side for 60 seconds and a picture was taken (Figure 2-3A). The vial was then cooled to $37^\circ C$ by placing the vial upright in a cell culture incubator, and a picture was taken after 60 seconds of the vial tipped on its side (Figure 2-3B). Similarly, the vial was then cooled to $20^\circ C$ (Figure 2-3C) and heated back up to $37^\circ C$ (Figure 2-3D). The methylcellulose solution is a gel phase at $60^\circ C$ and a solution phase at $20^\circ C$. At $37^\circ C$, the hysteresis property of the phase results in the methylcellulose solution to remain a gel at $37^\circ C$ during cooling but be a solution during heating. The inverse solution-gel phase transition and hysteresis property of methylcellulose solution is the foundation for the SLG process of controlling cell growth.

Figure 2-3 The inverse solution-gel transition property of methylcellulose (MC) solution and hysteresis effect of the phase of MC solution. Pictures A-D represents the points on the idealized curve. A) MC solution in gel phase after heating in $60^\circ C$ oven. B) MC solution remains in gel phase after cooling from $60^\circ C$ to $37^\circ C$ in cell incubator. C) MC solution in solution phase after cooling from $37^\circ C$ to $20^\circ C$ at room temperature. D) MC solution in solution phase after heating from $20^\circ C$ to $37^\circ C$ in cell incubator. Although B and D are at the same temperature (37°C), the viscosity and phase is shown to be different.
2.3 Previous Applications of Methylcellulose Gelation

The inverse solution-gel transition and hysteresis of methylcellulose solution have been used previously in applications such as forming detachable cell sheets and controlling fluid flow. Thirumala et al.[35] utilized the solution-gel transition and phase-transition hysteresis to culture monolayer cell sheets at an incubation temperature at 37°C that would detach for simple harvesting when the temperature decreased to 30°C. Tashiro et al.[34] and Ikeda et al.[36] used the reversible thermal gelation property of methylcellulose to create a switching flow valve. The temperature required to raise the methylcellulose solution in order to block the flow in a path was 55°C, and was achieved using a 1480 nm, 2 W laser. Similar to the methylcellulose gelation switching flow valve, the SLG method uses laser heating to cause methylcellulose gelation in order to control cell growth.

2.4 Laser Absorption in Methylcellulose

The design of the SLG system requires a laser to selectively heat methylcellulose solution to form a localized gel. To select the proper laser for selectively heating methylcellulose, several parameters must be considered. First, the absorbance of the selected wavelength laser in methylcellulose solution will affect the ability to locally heat the methylcellulose solution to the gelation temperature. Second, as the system is to be adapted to a standard inverted microscope common in biological laboratories, the transmission and reflection of the selected laser wavelength through internal microscope lenses and mirrors must be high to prevent loss of
power. The transmission of the laser through common cell culture microtiter plate materials such as glass and polystyrene must be high as well. While the transmission and reflectance of the various glasses and plastics are generally known, the absorption coefficient of methylcellulose solution is not a well-explored topic. However, as a first assumption, we use the absorption coefficient of water to model the response of methylcellulose solution. Methylcellulose or other semi-solid solutions used to culture cells are generally between 0.5%-2% by weight [23], [37], with the remaining solution being majority water and very a small volume of other cell culture additives. Using recorded absorbance data from Hale et al.[38], Figure 2-5 shows the absorption coefficient of water dependent at various wavelengths. The wavelength $\lambda = 1550$ nm was chosen as the laser for the SLG system because it has high transmission through glass and polystyrene, relatively high absorbance in water, and is an economically available and common wavelength used in telecommunications due to the low attenuation through glass and optic fiber [39].

![Absorption Coefficient of Water vs Wavelength](image)

**Figure 2-5 Absorption coefficient of water vs. wavelength. Dotted red line indicates the absorption coefficient where the wavelength $\lambda = 1550$ nm. Data retrieved from Hale et al. [40].**

For a more accurate model of heating due to laser absorbance, the change in absorbance of a material due to a change in temperature is also included in the model. Collins et al. explored the temperature dependence of the absorption coefficient ($\alpha$) of water from 0°C to 100°C from $\lambda = 700$ nm to $\lambda = 2100$ nm [41]. The data was taken from the $\lambda = 1550$ nm dataset and plotted with a
linear fit. Figure 2-6 shows the absorption coefficient in water at \( \lambda = 1550 \) nm plotted at 2°C, 20°C, 65°C, and 95°C. The resulting linear fit gives equation (2.2) – the effect of temperature \( T(\degree C) \) on the absorption coefficient \( \alpha (\text{cm}^{-1}) \) for water at a wavelength of 1550 nm. This equation is used in the heat transfer simulations described in Section 2.6.2.

\[
\alpha(T) = -0.0502T + 13.024
\] (2.1)

Figure 2-6 Temperature dependence of the absorption coefficient (\( \alpha \)) of water. Data retrieved from Collins, J [41].

2.5 Laser Focusing Optics

The laser beam must be focused down to an area small enough to target cells on the individual cell level so that adjacent cells are unaffected by the laser gelation. Antibody producing cells such as Chinese Hamster Ovary (CHO) cells have an approximate diameter of 12 – 14 \( \mu \)m [42]. A single mode, collimated circular beam can be focused using a thin lens, and the theoretical diffraction limited diameter of the focused spot \( d_{min} \) where the power falls to \( 1/e^2 \) (or approximately 86% of the power is contained) is defined by equation (2.2) where \( f \) is the focal length of the lens, and \( D \) the diameter of the incident collimated beam [43].

\[
d_{min} = \frac{2.44f\lambda}{D}
\] (2.2)

Using a \( \lambda = 1550 \) nm fibre-coupled laser collimated with a Thorlabs F280SMA-1550 collimating lens (Thorlabs, Newton, NJ), the initial diameter of the collimated beam has a diameter \( D = 3.6\)
mm. Table 2-1 shows the calculated minimum diffraction limited spot size using some available lenses for 1550 nm beam. The calculated values of $d_{min}$ show that it is possible to form spot sizes around the size of an individual CHO cell at wavelengths of 1550 nm using readily available equipment.

<table>
<thead>
<tr>
<th>Focusing Lens</th>
<th>$f$ (Focal Length, mm)</th>
<th>Calculated $d_{min}$ ($\mu$m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspheric Lens (Thorlabs A110TM-C)</td>
<td>6.24</td>
<td>6.56</td>
</tr>
<tr>
<td>Aspheric Lens (Thorlabs A260TM-C)</td>
<td>15.29</td>
<td>16.06</td>
</tr>
<tr>
<td>High-Power Focusing Objective</td>
<td>20</td>
<td>21.01</td>
</tr>
<tr>
<td>(Thorlabs LMH-10X-1064)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 2.6 Model of Laser Induced Localized Thermal Gelation of Methylcellulose

We developed a model of the heat transfer to estimate the size of the resulting gel when methylcellulose solution is heated using a focused laser spot. The methylcellulose solution is added on top of a polystyrene microtiter plate. The absorption of the heat from the focused laser spot provides the input heat flux. Thermal conduction through the methylcellulose solution and polystyrene microtiter plate is the output heat flux. The balance of these two fluxes determines the area in which the temperature is sufficiently large to form a gel.

#### 2.6.1 Input Laser Heat Flux

The heat source of the model comes from the absorption of the 1550 nm laser in the methylcellulose solution. The relation between absorption in a semi-transparent material and the intensity of the beam passing through is defined by the Beer-Lambert law [44]. As the laser light passes through the semi-transparent material, the intensity is attenuated and absorbed as heat. The Beer-Lambert law is described by equation (2.3) and equation (2.4) where $\alpha$ is the absorbance of the material, $\tau$ the transmittance, $I(z)$ the intensity of light as a function of depth through the material, and $I_0$ the initial intensity of light passing through the material.
Making the simplification that the light source is of a single wavelength and collimated within the thin layer of methylcellulose, the differential form of the Beer-Lambert equation as the laser travels through the thickness of the methylcellulose sample can be in the form shown in equation (2.5). The absorbance coefficient dependence on temperature, $\alpha(T)$, is also included in equation (2.5). This differential form of the laser absorption is used as the heat source in the finite element model used to simulate the size of the gelation area.

$$\frac{\delta I}{\delta z} = \alpha(\lambda, T) I(z) = \alpha(\lambda, T) I_0 e^{-\alpha(\lambda, T)z} \tag{2.5}$$

### 2.6.2 Output Heat Flux and Finite Element Model

Thermal energy absorbed from the laser heat source is dispersed via thermal conduction to the surrounding material. The area at which the majority of the laser intensity is absorbed in the methylcellulose results in a higher temperature, which spreads via conduction throughout the methylcellulose solution. The methylcellulose also loses heat by conduction through the polystyrene plate. The thermal spread due to conduction affects the gelation area.

A finite element model (COMSOL 5.3a, Burlington, MA) of the laser heating of methylcellulose solution was created to estimate the gelation area resulting from the input and output heat flux (Figure 2-7). The input heat source of the model was the laser absorption in methylcellulose. The heat conduction throughout the methylcellulose solution and polystyrene plate was used to calculate the resulting temperature distribution. The gelation area was determined from the resulting temperature distribution of the finite element model.

The geometry of the finite model included two contacting materials – the methylcellulose solution and the polystyrene microtiter plate. The differential absorption of the laser in methylcellulose (Equation (2.5)) was used as the heat source in the model. The laser was modeled as a collimated Gaussian beam as described in Equation (2.6), where $P$ represents the initial power of the laser, $w$ the waist of the beam where the power drops to $1/e^2$ of the peak power.
value, and $x$ and $y$ the Cartesian coordinates of the system. The time-varying temperature distribution of the model was calculated using Equation (2.7), where $Q$ is the heat source, $\rho$ is the density the material, $C_p$ is the specific heat capacity, and $k$ the coefficient of heat conductivity.

\begin{equation}
I_0(x, y) = \frac{P}{\pi w^2} e^{-\left(\frac{x^2+y^2}{w^2}\right)}
\end{equation}

\begin{equation}
\rho C_p \frac{\delta T}{\delta t} - \nabla \cdot (k \nabla T) = Q = \alpha(T) I(z)
\end{equation}

Simulations were run at varying values of the incident Gaussian beam power and waist size. Figure 2-8 shows an example of a single simulation result at power $P = 100$ mW and a beam waist of $w = 25$ µm. Figure 2-8A and Figure 2-8B show the thermal and isothermal planes at time $= 300$ ms. Figure 2-8C shows the temperature as a function of axial distance from the methylcellulose/polystyrene interface throughout the methylcellulose solution. Figure 2-8D shows the temperature as a function of radial distance at the interface of the methylcellulose solution and polystyrene microtiter plate. The data from the radial distance was used to calculate the diameter of the simulated gelation spot, which was defined as twice the radial distance at which the temperature of the thermal model was greater than a gelation temperature of $60^\circ$C. The calculated gelation spot sizes were then plotted along in Figure 2-9.
Figure 2-7 Finite element heat transfer simulation using COMSOL. A) Geometric model of system. The polystyrene plate contacting the methylcellulose solution is modeled. B) Generated mesh. The inner mesh closer to the peak of the Gaussian beam has a finer mesh to improve resolution. C) Intensity distribution of the laser source. The laser is modeled as a Gaussian at the interface of the methylcellulose and polystyrene and propagates through the methylcellulose. D) Simulated thermal surface of the model.
Figure 2-8 Example of simulation results. Power of $P = 100 \text{ mW}$, beam waist $w = 25 \mu m$ is shown. A) Thermal model at time = 300 ms. B) Isothermal planes at time = 300 ms. C) Temperature vs. depth axially through the centre of the methylcellulose solution. D) Temperature vs. radial distance from centre at the methylcellulose/polystyrene interface.
2.6.3 Estimation of Gelation Spot Size

The gelation spot diameter was calculated using the results of the finite element simulation. Gelation spot diameters for varying input power and beam waists are plotted in Figure 2-9. The simulation results indicate that with a smaller beam waist, the minimum required laser power to achieve gelation decreases. At higher powers, a larger beam waist will result in larger gelation area. While smaller gelation spot diameters can theoretically be achieved at various beam waist sizes, the gelation area can be more finely controlled with smaller beam waists, whereas with a larger beam waist, a small change in power will greatly affect the gelation spot diameter. The practical result of this is that a smaller beam waist will result in a wider operating range where the gelation spot diameter is small, allowing finer control of the gelation area to control cell growth using the SLG process. An ideal system design has a low slope in the operating range, where the gelation spot diameter can be controlled by controlling the power. However, this also shows an advantage of a thermal based system – if the power can be finely controlled, then the gelation area can be adjusted to very small areas.

Figure 2-9 Gelation spot diameters from simulated model at time = 300 ms. Simulations were run with varying beam waist size and varying laser power.
Chapter 3: Testing, Validation, and Automation

3.1 Overview

An experimental apparatus was created to test the ability of the SLG process to form localized gels in methylcellulose, and then to apply the SLG process to enable selective growth of antibody-secreting cells. To facilitate these goals, the experimental apparatus must be able to direct a focused infrared laser beam through an inverted microscope to a methylcellulose substrate. This apparatus must also retain the ability to perform fluorescence imaging in order to identify target cells. Section 3.2 describes the overall experimental apparatus and the specifics of the hardware devices including laser, motorized stage, camera, and other optical components. Section 3.3 then contains the experimental process to validate the thermal simulations of the gelation area. In Section 3.4, the SLG process is tested on live cells in vitro in order to experimentally show the selective growth of certain cells. Section 3.5 describes the method to detect and quantify the antibody production of cells. Section 3.6 and Section 3.7 discusses the custom software to control and automate the experimental apparatus.
3.2 Experimental Apparatus

The experimental apparatus to perform the SLG process involves a standard inverted fluorescence microscope with two excitation paths. The fluorescence optical path allows for the excitation and emission wavelengths for fluorescence imaging, which is necessary to detect antibodies secreted by the cells. The 1550 nm laser light path allows for the application of the laser onto the methylcellulose to cause thermal gelation.

Figure 3-1 Optical and control diagram of the experimental apparatus. The filter turret, mirror, and focusing lens/objective are all housed in a Nikon TI microscope. The camera, motorized stage and collimating lens are mounted to the microscope. A computer with custom software controls the camera, laser driver, and motorized stage position.

The system diagram of the experimental apparatus including all of the vital components is displayed in Figure 3-1. The fluorescence optical path begins with a fluorescence lamp (Nikon, Tokyo, Japan), which acts as the source for fluorescence excitation for the system. The light from the fluorescence lamp is collimated through the internal microscope lenses and passes through the fluorescence filter turret, which houses a mirror and EGFP filter set (Product Number 49002, Chroma Technology, Bellows Falls, VT) that isolates and reflects only the
excitation wavelength range for the secondary antibody used. The excitation wavelength passes through the 950 nm short-pass mirror (Product Number DMSP950R, Thorlabs, Newton, NJ), through a 4X Nikon CFI Plan Fluor microscope objective (Nikon, Toyko, Japan) and reacts with the antibody detection agent to output light at the emission wavelength (Section 3.5). The emission wavelength from the antibody detection agent passes back through the microscope objective, 950 nm short-pass mirror, and is isolated by the emission filter of the EGFP filter set to be detected by the camera (Product Number piA2400-17gm, Basler, Ahrensburg, Germany).

The 1550 nm laser path allows for the local gelation of the methylcellulose solution. An adjustable 2 W, 1550 nm infrared laser (Product Number LDX-3210-1550, RPMC Lasers, O’Fallon, MO) is fiber-coupled to a collimating lens (Thorlabs, Newton, NJ), then reflected through the 950 nm short-pass mirror towards the microscope objective. When using the infrared laser, a Nikon CFI Plan Fluor 20X microscope objective (Nikon, Tokyo, Japan) is used to focus the laser towards the target cells and methylcellulose solution. The sample containing cells and methylcellulose solution sits on top of a motorized stage (Product Number H117, Prior Scientific, London, UK) inside a temperature-controlled stage top incubator (Product Number UNO-T-H-CO2, Okolab, Pozzuoli, Italy) to take advantage of the phase hysteresis of methylcellulose solution around 37°C. The location of the sample is controlled by computer running custom software described in Section 3.6. The software controls the camera, infrared laser through a laser driver (Product Number 4308, Arroyo Instruments, San Luis Obispo, CA), and the 3-axis stage through a stage controller (Product Number ProScan III, Prior Scientific, London, UK).

The experimental apparatus is capable of imaging antibody-producing cells in bright field and fluorescence in order to detect cells and antibody secretion. The apparatus is also capable of using an infrared laser to induce the inverse solution-gel transition of methylcellulose vital to the SLG process in order to selectively inhibit arrest the growth of low-producing cells.

The components of the experimental apparatus are displayed in Figure 3-2 and Figure 3-3. The incubator sits on top of the motorized stage. The laser is mounted on a thermo-electric cooled mount (Product Number 264, Arroyo Instruments, San Luis Obispo, CA) to prevent the laser from overheating. The laser fiber attaches to the collimating lens connected to a 5-axis kinematic
mount (Model Number K5X1, Thorlabs, Newton, NJ). The 5-axis mount allows for slight adjustments in laser alignment and position and is mounted to the microscope using a custom 3D-printed adapter. The laser and thermo-electric cooled mount are controlled by separate controllers.

The alignment of the infrared laser is difficult as the infrared laser cannot be seen by the camera. A red 650 nm fiber coupled laser is used specifically for the alignment process. The red laser is coupled to the collimating lens in place of the infrared laser. To minimize the spot size, the adjustment knobs of the 5-axis kinematic mount are turned while the user views the camera output. The user adjusts the 5-axis kinematic mount until the red laser spot is as small as possible.

![Figure 3-2 Microscope and connected devices. The fluorescence filter set and 950 nm short-pass mirror are mounted internally as part of the microscope.](image)
Figure 3-3 Left: Infrared 1550 nm fiber-coupled laser and temperature controlled laser mount. Middle: fiber-coupled collimating lens and 5-axis kinematic mount for alignment. Right: Laser controller and temperature controlled laser mount controller.
3.3 Validation of Laser Gelation Area

The SLG experimental apparatus was tested to determine the minimal gelation area achievable by the system. The laser was pulsed at varying input currents and the resultant gelation spots were measured. The experimentally measured gelation spot diameters were then plotted on the same graph as the simulation gelation diameters to compare the theoretical heat transfer model with the experimental apparatus.

To experimentally measure the gelation area, 100 µL of 1% methylcellulose solution was plated into a multiple wells of a standard polystyrene 96-well microtiter plate. The 1550 nm laser was driven for 300 ms at various currents and the methylcellulose sample was imaged using standard bright field microscopy (Figure 3-4). For each current set point, the laser was activated ten times in different spots, and the gelation diameter for each spot was measured by taking the furthest points in the circular gelation area caused by the laser.

![Figure 3-4 Laser gelation spots around cells mixed in 1% MC using the experimental apparatus. The 1550 nm laser was driven at 2500 mA (497.5 mW) for 300 ms through a 20X objective. Gelation spots have an average measured diameter of 103.2 µm.](image)
The experimentally measured gelation diameters were plotted on top of the simulated gelation diameters (Figure 3-5). As the laser power increased, the measured gelation diameter increased as well, following a similar trend as the simulated results. A current set point below 2500 mA did not result in any visible gelation areas. The minimum diameter of the gelation was measured to be 103.2 µm when the laser was driven at 2500 mA for 300 ms through the 20X objective. The average and standard deviation of the gelation spot diameters for each current input tested are listed in Table 3-1.

![Figure 3-5 Experimental and simulation results of gelation spot diameter as a function of laser power with the laser driven for 300 ms. Simulation curves are plotted with varying beam waist sizes. Error bars represent ± SD.](image-url)
Table 3-1 Measured gelation spot diameter experimental data for various current inputs using 20X objective.

<table>
<thead>
<tr>
<th>Current Input (mA)</th>
<th>Power From Diode (mW)</th>
<th>Estimated Power After System Losses (mW)</th>
<th>Average Gel Spot Diameter (μm)</th>
<th>Standard Deviation (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2500</td>
<td>497.5</td>
<td>81.4</td>
<td>103.2</td>
<td>9.0</td>
</tr>
<tr>
<td>3000</td>
<td>635.0</td>
<td>103.9</td>
<td>131.9</td>
<td>8.1</td>
</tr>
<tr>
<td>3500</td>
<td>772.5</td>
<td>126.4</td>
<td>187.0</td>
<td>14.6</td>
</tr>
<tr>
<td>4000</td>
<td>900.0</td>
<td>147.3</td>
<td>220.5</td>
<td>15.9</td>
</tr>
<tr>
<td>5000</td>
<td>1160.0</td>
<td>189.9</td>
<td>240.2</td>
<td>31.0</td>
</tr>
</tbody>
</table>

The power delivered to the methylcellulose solution is used to plot the experimentally measured gelation spot diameter in Figure 3-5. The input power to the methylcellulose solution was calculated by estimating the power loss of the laser along the infrared optical path of the experimental apparatus. The initial power emitting from the laser diode is known for a given current from the manufacturer’s datasheet [45]. Beginning from the laser diode, the components that contribute to power loss as the infrared laser beam travels through the system are the optic fiber coupling, collimating lens, dichroic 950 nm short-pass mirror, microscope objective, and polystyrene well plate. For a wavelength of 1550 nm, the power remaining after the 1550 nm laser beam passes through or is reflected at each component is listed in Table 3-2. The total calculated power remaining after losses is 22.9%, the combination of all of the losses through the system. The third column in Table 3-1 was calculated by multiplying the power from the laser diode by 22.9%.

Table 3-2 Transmission and reflectance of the 1550 nm laser through various components in the system

<table>
<thead>
<tr>
<th>Component</th>
<th>Power Loss From Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optic Fiber Coupling</td>
<td>75% (manufacturer’s data)</td>
</tr>
<tr>
<td>Collimator (transmission)</td>
<td>99.875% [46]</td>
</tr>
<tr>
<td>Dichoric 950 nm short-pass mirror (reflectance)</td>
<td>99.875% [46]</td>
</tr>
<tr>
<td>Nikon Plan Fluor 20X (transmission)</td>
<td>35% (estimated from [47])</td>
</tr>
<tr>
<td>Polystyrene Microtiter Plate (transmission)</td>
<td>90% [48]</td>
</tr>
<tr>
<td><strong>Total Power Remaining After Losses</strong></td>
<td><strong>22.9%</strong></td>
</tr>
</tbody>
</table>

Differences between simulated and experimental gelation spot diameter could arise from various sources including the estimated power loss of the system, the laser being focused as opposed to the simulation assuming a collimated laser throughout the methylcellulose solution, optical alignment, using the thermal properties of water in place of methylcellulose in the simulation, an
assumed gelation temperature of 60°C, and the absence of convection or radiation losses in the heat transfer model. In addition, the manufacturer of the laser advised that operating the laser at a current below 2500 mA would not output the full power as the datasheet listed. This shows the practical limitation of the experimental apparatus and why decreasing the spot size is still important – as the laser power output is not easily controlled at the lower range of operation, it becomes difficult to create smaller gelation spot diameters.
3.4 Validation of Selective Cell Growth

Figure 3-6 Selective cell growth using the SLG process of B13-24 cells plated in 1% MC/IMDM media. The dotted yellow circle indicates the cell on which the laser was applied. Top: 3 individual cells plated on initial day 0. Bottom: One day later, two non-lasered cells have started to divide, while the lasered cell has not.

To confirm the SLG process is able to selectively arrest the growth of specific cells, we plated B13-24 CHO cells in 1% methylcellulose solution and applied the laser to certain cells only. The laser was applied with a current set point of 2500 mA for a duration of 300 ms. The cells were then cultured and the cell growth was monitored over time. Cells that had the laser applied were observed to stop the growth process, where as other cells started to divide (Figure 3-6).
Figure 3-7 Selective cell growth of UC13-EGFP cells (green) and UC3-mCherry (red) cells plated in the same well in 1% methylcellulose solution. A) Control sample well on day 1. UC13-EGFP and UC3-mCherry cells were plated in the same initial well. B) Control sample well on day 7. The well from picture A was allowed to grow for 7 days. C) Experimental sample well on day 1. UC13-EGFP and UC3-mCherry cells were plated in the same initial well. The 1550 nm laser was then applied to all UC3-mCherry cells D) Experimental sample well on day 7. After applying the laser to all UC3-mCherry cells, only the UC13-EGFP cells propagate. (Scale bar = 50μm)

The SLG process for selective cell growth was also validated by plating two cell lines in the same well, and then applying the laser to only selectively gel one cell line. UC13-EGFP (green) and UC3-mCherry cells (red) were mixed with 1% methylcellulose solution and plated into the same well. The wells were imaged using a fluorescence microscope for EGFP and mCherry to view both cell lines. We then applied the laser to the red UC3-mCherry cells, and cultured the wells for seven days. After seven days, the wells were imaged once again. In the control well, both cell lines were visible (Figure 3-7B). In the experimental well where the laser was applied onto red cells, only the green UC13-EGFP cells remain after seven days of culture (Figure 3-7D). This experiment validates that the SLG process can prevent certain cells in a mixed population from growing.
3.5 Detection of Secreted Antibody in Methylcellulose Media using a Fluorescently Conjugated Secondary Antibody

In order to use the SLG method to select antibody-secreting cells producing high amounts of antibodies, a method is required to distinguish between high-producing cells and low-producing cells plated in the same well. Unlike the previous experiment in Section 3.4 with two separate cell lines easily distinguishable, there is only one antibody-secreting cell line plated into each well. The antibody-secreting cells do not have intracellular fluorescently conjugated proteins to distinguish high and low-producing cells, and there are no morphological differences of cells producing differing amounts of antibodies.

The detection of secreted antibody prior to the laser gelation process is achieved using a fluorescently-conjugated secondary antibody. A secondary antibody is an antibody molecule that binds to the primary antibody secreted by the cell. Fluorescently-conjugated secondary antibodies are commonly used in immunolabeling to allow for the indirect detection of the presence and quantity of the primary antibody, as the primary antibody secreted by the cell do not have an innate fluorescent marker or tag. The use of a fluorescently conjugated secondary antibody as a method to detect antibody secretion levels is a common method for selecting antibody-secreting cells in various methods such as semi-solid cloning and FACS. Caron et al. [26] use a secondary antibody targeted against IGF-E5 producing cells plated in methylcellulose media to determine high-producing colonies prior to colony picking. The specificity of the method is equal to the specificity of the secondary antibody used.
Cells are normally grown in a liquid cell culture media. By plating cells in methylcellulose solution instead of liquid media, the cells grow into distinct colonies and also retain antibodies secreted from the cells in the area around the cell. To detect the secreted antibody within the methylcellulose solution, a fluorescently-conjugated secondary antibody is mixed into the methylcellulose solution along with antibody-secreting cells prior to plating. A fluorescently-conjugated secondary antibody dispersed in the methylcellulose media displays brighter concentrations around the producing cell colony. With this system, as long as a fluorescently-conjugated secondary antibody targeting the primary antibody can be made, then it is theoretically possible to detect for a specific antibody secreting from a cell colony regardless of what antibody is being secreted. Figure 3-8 describes the principle of the detection of antibody secreted from a cell using a fluorescently conjugated secondary antibody. Figure 3-9 shows the use of the secondary antibody CloneDetect Human IgG (H+L) Specific, Fluorescein (Molecular Devices, Sunnyvale, CA) for the detection and quantification of IgG4 secreted by B13-24 CHO cells (ATCC, Manassas, VA).
Figure 3-9 Detection of secreted human IgG4 from B13-24 cell colonies in solution containing 1% MC and 1% CloneDetect Human IgG (H+L) Specific, Fluorescein (Molecular Devices, Sunnyvale, CA). Left: Brightfield image of two separate cell colonies. Right: EGFP image (false colour applied), showing the upper colony is secreting IgG4 while the lower colony is not.

In limiting dilution or single cell plating methods, a single cell must be grown to a sufficiently large enough culture before average productivity can be measured by methods such as ELISA. Measurement by ELISA requires the wells to have enough antibodies produced to meet the minimum range of detection, which can take many days to more than a week depending on the cells. Growing the cells into separate colonies in methylcellulose solution containing a fluorescently conjugated secondary antibody allows for the detection of high-producing colonies earlier in the process, reducing the overall timeline for cell selection.
3.6 Software Development and User Interface

A computer program was developed for the experimental apparatus to be able to take images from the camera, analyze the images for cells and antibody-production levels, move the motorized stage to center the laser on low-producing cells, and activate the laser to selectively gel the methylcellulose. The user interface (UI) is designed to allow the user to view the camera output while being able to change parameters for the camera (gain, exposure) and laser (current set point, continuous wave or pulse-width operation), and allow the user to manually operate the camera, laser, and motorized stage simultaneously. The computer program was written in C# and the UI was written in Windows Presentation Foundation (WPF) using the markup language XAML to define interfacing elements.

Figure 3-10 Overview of C# program to control the process and all peripheral hardware devices.
Figure 3-10 shows the user interface for the C# program created to control the process with the sections of the program labeled in boxes 1-3. Section 1 of the program has five headers – Main Control, Serial, Stage, Position, and Image. The header selected determines the controls appearing in section 2. Section 2 houses all the control fields and buttons for the user to interface with the connected hardware. Section 3 displays the camera output in real time or results of the image processing steps.

The user interface for the C# control software consists of 5 selectable panels including Main Control, Stage, Serial, Position and Image (Figure 3-11). The Main Control panel (Figure 3-11A) allows the user to connect to the stage, the laser controller, and the camera, and to adjust parameters for the camera and laser. The current set point of the laser can be set in units of milliamps. The laser can be toggled on and off in continuous wave format, or can be operated in a Pulse Width Modulation (PWM) mode. In PWM mode, the period and duty cycle can be set by the user. A user inputted number of cycles can be fired to test the PWM settings, or for manual control of the laser. The camera can also be connected through gigabit Ethernet and controlled...
through the main panel. The user can take a single snapshot with the camera, or more commonly operate with continuous stream of images to view the experiment in real time. The exposure and gain and also be controlled by the user depending on the sample being viewed.

The Stage control panel (Figure 3-11B) includes many parameters relating to the motorized stage. The primary operation of the Stage control panel is to operate the scanning and stitching of images larger than one field of view of the camera and objective. For the scanning method, the number of fields in X and Y direction, as well as the objective being used to scan is specified and using the Scan button the stage and camera will automatically save an image of the current field, move to the next field, and repeat until images of all fields have been saved. An image stitching ImageJ plugin[49] is then called to perform the stitching and the resulting stitched image can be loaded back into the program. Additionally, stage position is queried on an interval and the communicated position is displayed in the Stage Position section. The specific position desired can also be controlled using the Go To section.

The Serial control panel (Figure 3-11C) includes the diagnostics for serial communication with the stage controller and laser. Commands sent to and received from both devices are shown for debugging purposes. Single commands can be sent to each device for testing purposes.

The Position control panel (Figure 3-11D) contains stage position coordinate info of the target well. When a well is scanned, each pixel of the resulting stitched image must be calculated in relation to the original position of the stage for future image processing steps. During the scanning process, the program records the (X, Y) location of each image during acquisition, and uses the (X, Y) information to calculate the stage position of each pixel in the resultant stitched image. The position control panel displays the well position info of the top left (X1, Y1) and bottom right (X2, Y2) position of the stitched image. The lens or objective used to scan the laser is selected in this panel, and laser offset due to misalignment can also be corrected for.

The Image control panel (Figure 3-11E) includes individual buttons to load bright field (BF) and fluorescence images and to start the image processing pipeline described in Figure 3-12. Buttons are available to load stitched images back into the program. The fluorescence threshold for antibody secretion detection can be manually set, and the number of colonies to keep after the laser process can also be changed.
3.7 Image Processing and Automation

![Image Processing Pipeline Diagram]

Figure 3-12 Image processing pipeline to detect and rank cell colonies secreting antibodies.

An image processing pipeline was developed to automate the detection of high-producing cell colonies and the elimination of low-producing cell colonies. The purpose of the image processing pipeline is to take a bright field (BF) stitched image and a fluorescence (EGFP) stitched image as inputs, detect the location of cells and small cell colonies, quantify the antibody produced by each cell, and output the location of the colonies that are to undergo laser methylcellulose gelation.

The image processing pipeline (Figure 3-12) was implemented in software using EmguCV, a .NET wrapper for the Open Source Computer Vision Library (OpenCV) [50]. Using the BF image, cell colonies are detected using the Canny edge detector [51] with the upper and lower thresholds automatically determined using Otsu’s method [52]. A contour detection algorithm [53] is used to detect the centroid location (X, Y) and create a bounding box around the cell colony. The fluorescence image is run through an adaptive threshold to detect secreted antibody. Each centroid and bounding box area ($A_{colony}$) detected from the BF image is used to count the number of pixels of secreted antibody ($N_{ab}$) detected after the adaptive threshold from the
fluorescence image. The colonies are then ranked using the ratio \( N_{ab}/A_{colony} \). The top ranked colonies are preserved and the remaining (X, Y) locations of centroids of the remaining colonies were used to move the motorized stage to center the laser on each undesired colony.

The imaging pipeline from acquisition through to determining the high-producing colonies can be seen in the following figures. Figure 3-13 shows the stitched images of a well under BF and EGFP filters. Figure 3-14 shows the results of the combined cell detection in BF and antibody secretion in EGFP. The red dots and rectangles show the centroids and bounding boxes detected. The detected secreted antibody is displayed in white. Although there can be falsely detected cell colonies, the false detections will be removed once the cell colonies are ranked based on amount of protein secreted. Clumped secondary antibody on debris also appears in the original EGFP image.

![Figure 3-13 Image acquisition. Stitched bright field (BF, left) and fluorescence (EGFP, right).](image-url)
Figure 3-14 Results from image processing steps of combined cell detection and antibody secretion detection
Figure 3-15 A) One well in 96-well plate after high and low-producing cell colonies are detected using custom image processing software. High-producers are detected by software and displayed a red bounding box. Examples of high-producing and low-producing colonies are shown in yellow and blue dashed rectangles respectively. B) Magnified view of high-producing colony, software detected IgG4 shown in white. C) Magnified view of high-producing colony, multichannel BF/EGFP image prior to software IgG4 detection. D) Magnified view of low/non-producing colonies. Low amounts of IgG4 detected by software. E) Magnified view of low-producing colony, multichannel BF/EGFP image prior to software IgG4 detection.

Figure 3-15 shows an example of high-producing and low-producing cell colonies detected by the image processing software. The top producing cell colonies remain after ranking of cell colony productivity (Figure 3-15A). An example of a high producing cell colony with the antibody secretion detected (Figure 3-15B) and the original BF/EGFP image of the colony is also shown (Figure 3-15C). One of the many low or non-producing cell colonies are also displayed (Figure 3-15D, E). The top three cell colonies are removed from the list of cell colony centroids prior to sending the centroid coordinates of the undesired cells to the stage controller.
Table 3-3 Excerpt of the list of cell colonies and protein secretion (EGFP) detected in Figure 3-14. All values shown are normalized to the highest number in each column. The top 3 ranked cell colonies are then removed from this list before the remaining centroids are passed to the motorized stage.

<table>
<thead>
<tr>
<th>Rank</th>
<th>Area</th>
<th>Protein Secretion (EGFP)</th>
<th>EGFP Area</th>
<th>Centroid (X, Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.11</td>
<td>0.15</td>
<td>1.00</td>
<td>(1606, 1099)</td>
</tr>
<tr>
<td>2</td>
<td>0.97</td>
<td>1.00</td>
<td>0.77</td>
<td>(3237, 798)</td>
</tr>
<tr>
<td>3</td>
<td>0.23</td>
<td>0.18</td>
<td>0.57</td>
<td>(1592, 1134)</td>
</tr>
<tr>
<td>4</td>
<td>0.11</td>
<td>0.04</td>
<td>0.25</td>
<td>(2080, 3959)</td>
</tr>
<tr>
<td>5</td>
<td>0.06</td>
<td>0.01</td>
<td>0.15</td>
<td>(1806, 4106)</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Table 3-3 shows an excerpt of the recorded and calculated parameters of the cell colonies detected in Figure 3-14. The area of the bounding box and the protein secretion detected in the fluorescence spectrum using the EGFP filter are recorded for each cell colony. The cell colonies are then ranked by the ratio of EGFP divided by the bounding box area. The top 3 cell colonies removed from this list, and the remaining cell colony centroid positions are used to calculate the positions that the stage must move to before activating the laser. Equations (3.1) and (3.2) are used to convert the centroids in pixel value shown in Table 3-3 to stage stepper motor values \((x_{stage}, y_{stage})\) which have a unit of 0.04 \(\mu m\) per step. Pixel to conversion constants \(C_x\) and \(C_y\) were calculated by taking single pictures of a hemocytometer on the microscope, and correlating the pixel values of the resultant pictures with the known dimensions of the hemocytometer. Offset values \(x_{offset}\) and \(y_{offset}\) are input using the Set Laser Offset \((X, Y)\) button in the Position panel in Figure 3-11, and are used to compensate for any misalignment in the laser. The two opposite corners of the stitched image \((x_1, y_1)\) and \((x_2, y_2)\) are determined by the software during the image acquisition process.

\[
x_{stage} = C_x \frac{x_{pixel}}{(x_2 - x_1)} + x_{offset} \tag{3.1}
\]
\[ y_{\text{stage}} = C_y \frac{y_{\text{pixel}}}{(y_2 - y_1)} + y_{\text{offset}} \] 

(3.2)

After conversion of the centroids \((x_{\text{pixel}}, y_{\text{pixel}}) \rightarrow (x_{\text{stage}}, y_{\text{stage}})\), the remaining list of positions is sent to the stage controller. The stage then sequentially travels to each point in the list at a stage speed of 3 mm/s. Upon reaching each \((x_{\text{stage}}, y_{\text{stage}})\) coordinate, the laser is pulsed at 2500mA (497.5 mW) for 300 ms. The entire image acquisition, image processing, and laser scanning step takes approximately 5 minutes per well of a 96-well plate.
Chapter 4: Materials and Methods

This chapter describes all the materials and methods used in the experiments in this thesis. The methods described are typical of standard biological protocols. The preparation of media used for regular cell culture and the methylcellulose solution required for SLG, as well as the cell line used in the experiments are described. Methods for plating and supernatant gathering used are explained. A brief description of the ELISA method to measure the antibody concentration and perform statistical analysis is included.

4.1 Solution Preparation

4.1.1 Isocove’s Modified Dulbecco’s Medium (IMDM) Liquid Media

Liquid Isocove’s Modified Dulbecco’s Medium (IMDM) is required for culture of cells. To prepare, add 50 mL 0.22 μm filtered heat-inactivated fetal bovine serum (FBS) (Thermo Fisher, Watham, MA) and 1mL 100X concentrated penicillin-streptomycin (P/S) (Thermo Fisher, Watham, MA) to 450mL of IMDM containing 4mM L-glutamine, 4500 mg/L glucose, and 1500mg/L sodium bicarbonate (ATCC, Manassas, VA).

4.1.2 Methylcellulose (MC) / IMDM Solution

To prepare a 2.5% methylcellulose (MC) solution, 950 g of distilled water and 24.81 g of Sigma MC powder (M0512, 4,000 cP, Sigma-Aldrich, St. Louis, MO) were autoclaved separately at 121°C for one hour. After autoclaving, using sterile technique, the MC powder was added to the distilled water while slowly stirring with a metal spoon. Magnetic stirring bars were added prior to sealing and the jar was placed on a magnetic stirrer and allowed to cool to room temperature (~20°C). After cooling, 17.7 g of IMDM powder (Sigma-Aldrich, St. Louis, MO) was added to the MC solution, stirred for an additional two hours and left overnight at 4°C.

To dilute to a final working concentration of 1% MC, 20 g of the 2.5% MC/IMDM solution, 23.7 mL of 1x IMDM media (ATCC, Manassas, VA), 5 mL heat-inactivated fetal bovine serum (FBS) (Thermo Fisher, Watham, MA), 800μL of 7.5% sodium bicarbonate solution (Thermo Fisher Scientific, Waltham, MA) and 500μL of 100X concentrated Penicillin/Streptomycin (P/S) solution (Thermo Fisher, Watham, MA) are mixed using a vortex mixer on high and left
overnight at 4°C to disperse any bubbles. The next day, the MC/IMDM solution is filtered through a 0.45µm filter to remove any residual clumps and debris.

4.2 Antibody-Secreting Cells

B13-24 (Catalog Number CRL-11397, ATCC, Manassas, VA) Chinese Hamster Ovary (CHO) cells were grown in Isocove’s Modified Dulbecco’s Medium (IMDM) (ATCC, Manassas, VA) containing 4 mM L-glutamine, 4500 mg/L glucose, and 1500 mg/L sodium bicarbonate supplemented with fetal bovine serum (FBS) (10%v/v) and 1X penicillin-streptomycin (P/S) (Thermo Fisher Scientific, Waltham, MA). B13-24 is a CHO cell line that is capable of producing up to 100µg/L of humanized immunoglobulin IgG4 at a rate of 25 pg/cell/day [54].

4.3 Plating in Methylcellulose Solution

To plate B13-24 cells in 1% MC/IMDM solution, 1000 cells were added to 1 mL of 1% MC/IMDM solution with 10 µL CloneDetect Human IgG (H+L) Specific, Fluorescein (Molecular Devices, Sunnyvale, CA). The solution was mixed slowly using a 1 mL syringe with 16g needle, taking care to prevent bubbles from forming. 100 µL of the solution was dispensed into the inner wells of a 96 well flat-bottomed polystyrene plate. The 96-well plate was centrifuged at 530 x g for 5 minutes and incubated at 37°C, 5% CO₂ for two days.

4.4 Cell Culture and Supernatant Retrieval

Cells were incubated in a humidified incubator at 37°C, 5% CO₂. After incubating in the MC/IMDM media for a varying amount of days based on the experiment, 200 µL of IMDM media described in section 4.1.1 was added to each well containing cells. The supernatant was sampled and media refreshed at multiple time points to quantify the antibody level.

4.5 Antibody Level Measurement by ELISA

Measurement of the antibody level produced by the grown cells was done using enzyme-linked immunosorbent assay (ELISA). An IgG4 Human ELISA Kit BMS2095 (Thermo Fisher, Waltham, MA) was purchased to verify the antibody secretion titer. The kit can measure human IgG4 antibody with a range of 9.4-600 ng/mL with a sensitivity of 0.1 ng/mL. A full description
of the ELISA procedure can be found in the technical data sheet [55], but a brief description of the ELISA procedure is as follows:

First, supernatant was sampled from each well of the experimental cultures. A 96-well plate pre-coated with anti-human IgG4 antibody was provided in the ELISA kit. The plates are washed using the wash buffer, then a serial dilution of the provided 1,200 ng/mL antibody standard was performed on the first two columns of the 96-well plate to define a standard curve. A small sample of each supernatant sample is diluted with the assay buffer to a final volume of 100 μL (e.g. if the sample is to be diluted 1:5, then 20 μL of supernatant is diluted with 80μL of assay buffer.) 50 μL of horseradish peroxidase (HRP) is added to each well of the 96-well plate and the plate was shaken for one hour. After shaking, 100 μL of tetramethyl-benzidine substrate solution was added to each well and incubated at room temperature for 30 minutes while avoiding exposure to light. 100 μL of stop solution was then added to each well to stop the reaction. The absorbance of each well was read using a SpectraMax 190 spectrophotometer (Molecular Devices, Sunnyvale, CA) using 450 nm as the primary wavelength and 650 nm as the reference wavelength. Each well sample was sampled and analyzed twice and averaged, ensuring that neither value differed by more than 20% of the mean value as according to the ELISA kit guidelines. The measured absorbance in the first two columns of the 96-well plate was used to determine a standard curve using a 5 parameter logistic regression. The concentrations of the experimental samples were calculated using the standard curve and the absorbance in each of the sample wells.

4.6 Statistical Analysis

Data was plotted in GraphPad Prism v7.0 (GraphPad Software, San Diego, CA) and analyzed using the two-tailed unpaired Student’s t-test with Welch’s correction.
Chapter 5: Experimental Results

5.1 Experimental Overview

We used the SLG process to selectively arrest the growth of low-producing cells in order to increase the productivity of an antibody-producing cell line. The overall experimental plan is described in Figure 5-1. B13-24 CHO cells secreting humanized IgG4 were mixed with a 1% methylcellulose solution containing a fluorescently labeled secondary antibody. The methylcellulose solution containing cells is then plated into a 96-well plate. Two days later, the well was scanned in bright field and fluorescence to detect cell colony size and secreted antibody. The software ranked the colonies based on sized and amount of antibody produced, and then determines the high-producing cell colonies to be retained. A laser is then pulsed over the remaining colonies to arrest their growth. The selected high-producing cells are allowed to grow and liquid IMDM cell culture media is added to each well and incubated for an additional twelve days. After incubation, the liquid supernatant is gathered by a pipette for further antibody quantification by ELISA, and the culture is then expanded into a larger container.

Figure 5-1 Experimental procedure from plating cells to expansion of culture. 1) B13-24 CHO cells are mixed into a solution containing 1% MC, 1% CloneDetect Human IgG (H+L) Specific, Fluorescein. 2) The solution from step 1 is plated into the inner wells of a 96-well plate. 3) Each well is scanned in bright field and fluorescence to detect cells and secreted IgG4. 4) Colonies are ranked based on size and antibody secretion. 5) The top producing cells are kept and the laser is scanned over the remaining cells. 6) Liquid media is added to each well and the remaining cells are incubated and allowed to grow out. 7) The supernatant containing secreted antibody is gathered for analysis using ELISA 8) The culture is expanded into increasingly large containers.
5.2 Selecting Cells based on Antibody Production

To assert that the SLG selection can be used to improve productivity of antibody-secreting cells, we used this method to selectively enrich for both high-producing cells in one population and low-producing cells in another. The resultant two selected populations were compared with control cells that remained undisturbed (no laser applied) after plating. We hypothesized that selection for high-producer cells would expand a population with greater antibody production than the control, while selection for low-producer cells would have lower antibody production than the control.

![Timeline for experiment to select high producing cell colonies. Numbers indicate days elapsed during experiment.](image)

A key advantage of the SLG process is that the entire process can be completed within three days. However, we subsequently monitored the cells both in methylcellulose and liquid culture over twelve days, prior to assessment of antibody production using ELISA, as depicted in Figure 5-2. B13-24 cells were plated in 1% methylcellulose solution containing a fluorescently-conjugated secondary antibody on day 1. After two days of culture, the SLG process is performed. For the high-producer group, the software ranked the cell colonies based on antibody productivity and colony size, and the top three ranked colonies were preserved, while the remaining colonies were selectively arrested by laser-induced gelation. In the low-producer group, the bottom three ranked cell colonies were kept and the laser was applied on the
remaining cells. Cells were then cultured for six days in the methylcellulose solution, with manual observation every day to ensure only selected colonies were growing. On day 9, 200 µL of liquid IMDM media was added to each well. Cells were then cultured for an additional 6 days. On day 15, a sample of each well was taken for further antibody quantification by ELISA.

As illustrated in Figure 5-3, by allowing for six days of outgrowth in methylcellulose culture, we could observe that only the preserved cell colonies were growing and secreting antibody. The antibody secretion was determined using a fluorescently-labeled secondary antibody, which aggregated around the preserved colonies. Furthermore, after subsequent culture of these cells in liquid IMDM media, representing 15 days total growth, ELISA was performed to compare IgG4 concentration of control cultures with cultures selecting for high-producing cells as well as cultures selecting for low-producing cells (Figure 5-4). Control wells, having undergone no gelation, had a mean IgG4 concentration of 688.5 ng/mL with a standard deviation of ±124.5 ng/mL. Wells in which the high-producing colonies were preserved had a mean IgG4 concentration of 2039.3 ng/mL with a standard deviation of ±889.8 ng/mL. This represented a 2.96-fold increase in mean IgG4 concentration compared to control and was significant by two-tailed unpaired t-test using Welch’s correction (N = 5, p = 0.0267). Wells in which the low-producing colonies were preserved had an average IgG4 concentration of 222.3 ng/mL with a standard deviation of ±83.4 ng/mL. This represented a 3.58-fold decrease in mean IgG4 concentration compared to control and was also significant (N = 5, p = 0.0002). Together, these results show that the SLG process in methylcellulose solution can be used to isolate a high-producing subpopulation in well containing only a single cell line with cells secreting varying amounts of antibody, and the resultant cell line has an overall higher antibody productivity.
Figure 5-3 – Two cell colonies (A, B) after laser process and culturing in MC for 6 days. Wells were checked every two days to ensure only desired cell colonies were growing.

Figure 5-4 – IgG4 Concentration as measured by ELISA from supernatant sampled on day 15 in the 96 well plate. Wells in which top 3 high-producing cell colonies were kept resulted in a mean antibody concentration higher than control wells ($N = 5$, $p = 0.0267$). Wells in which 3 low-producing cell colonies were kept resulted in a mean antibody concentration lower than control wells ($N = 5$, $p = 0.0002$).
5.3 Expansion and Stability

To establish the long-term stability of the enhanced antibody production rate for the cell line developed using the SLG process, we monitored the antibody production rate for 25 days. Figure 5-5 shows the timeline of the experimental process.

![Timeline](image)

**Figure 5-5** Timeline for expansion and stability experiment. Red circles indicate days that a 24-hour batch media was sampled for analysis by ELISA. Numbers indicate days elapsed during experiment.

Cells were plated on day 1 and the screening and laser process was performed on day 3. Colonies were grown in methylcellulose solution for an additional two days to confirm the selection process. On day 5, the culture was supplemented with 200 µL of liquid IMDM media and then grown to confluence within the well. On day 14, the cells were transferred from the original 96-well plate to a 12-well plate (area = 4 cm²) to prevent overgrowth. By day 18, the cells had grown to near-confluence so they were enumerated by hemocytometer and seeded at equal cell density to a new 12-well plate. This culture was then expanded to a 6-well plate (area = 9 cm²) on day 19, and then expanded to a T-25 (area = 25cm²) flask on day 20. Samples of the supernatant were taken on days 8, 13, 19, 20, and 25 for analysis by ELISA.
ELISA measurements of the antibody production rate sampled from supernatant on different days are shown in Figure 5-6. Early samples of the cell culture supernatant showed that the control group had a higher total productivity compared with the other two experimental groups. However, from day 12, as the culture was allowed to expand, the wells in which the high antibody-producing cells were preserved had increased mean antibody productivity compared with both the control group and the low antibody-producer group. Each experimental group had a sample of N = 3 for a total of 9 wells. All sampling was from a static 24-hour batch culture. These results suggest that the high-producing cell line retained their increased IgG4 antibody production rate after 25 days of culture (N = 3, p < 0.1).
5.4 Discussion

Optimal production cell lines are vitally important for the development of monoclonal antibodies as potential therapeutics because significant amount of material is required for *in vitro, in vivo*, and ultimately, clinical testing. By selectively enabling the growth of high-producing cells, the SLG process can increase the overall productivity of the production cell line and thereby dramatically shorten timelines for research and commercialization. Compared to other methods, the SLG process provides a shorter timeline for cell line development, allowing for high-producing cells to be selected in as little as two days after plating. The earlier selection of high-producing cells prevents the fast-growing, low-producing cells from dominating the cell culture. Additionally, the SLG procedure is non-contact process, which dramatically reduces the risk of contamination. The power density for SLG to arrest cell growth is much lower than alternative laser-based methods. The SLG procedure uses a standard microtiter plate, without requiring specialized plates or additional staining procedures. The stability of the increased antibody production of cells selected using the SLG process is a promising result, as other selection methods such as gene amplification can be susceptible to a loss in productivity over time. The SLG process is also amenable to automation, and further improvements to the experimental apparatus could dramatically increase the speed and throughput of the process.

While SLG provides a rapid platform for both increasing yield and stability of production in antibodies, this work also creates opportunities to refine and adapt this process further. Firstly, while this study demonstrated improved antibody production over 25 days, cell line systems must be expanded tremendously in order to produce industry-scale antibodies. A key question remains whether the cell lines will retain their high level of productivity throughout this extended production process. Secondly, while we achieved precise gelation of cells, potential high-producing cells were omitted from selection due to their proximity to other cells. Improvements to the experimental apparatus in order to decrease the gelation area could overcome this limitation and potentially increase the throughput of the system, making it more attractive for commercial applications. Finally, the SLG process could be applied to a range of existing hybridoma and production cell lines to enhance their productivity, or to other cell lines to select for desirable characteristics or to enhance cell line clonality.
Chapter 6: Conclusion

6.1 Summary of Results

This work developed a novel technology called Selective Laser Gelation (SLG) that selectively inhibits the growth of low-producing antibody-secreting cells in methylcellulose solution. The SLG process relies on the inverse gel forming property of methylcellulose to create localized gels to selectively arrest the growth of target cells. The gelled regions are then retained due to gelation hysteresis after the laser is removed.

This thesis described a heat transfer model and simulation created using the material properties of methylcellulose solution. An experimental apparatus capable of localized laser gelation was designed and created using the results of the simulation, and was used to validate the thermal model and simulation. Selective cell growth using the SLG method was validated using fluorescently-conjugated bladder cancer cell lines. Custom software was developed to allow the user to interface with the hardware devices, and automate the antibody secretion detection process. Finally, the entire system was applied to select antibody-secreting cells producing high amounts of antibodies.

The SLG process has been demonstrated to be able to select high and low antibody-producing cell colonies based on the detection of a secreted antibody detected by a fluorescent secondary antibody. Experimental results verified by ELISA showed that high-producing cell colonies selected using the SLG method resulted in a 2.96-fold increase in mean antibody production over control samples after 15 days. Samples in which low-producing cells were selected had a 3.58-fold decrease in mean antibody production over the control group. The study of the stability of the antibody productivity in the selected cells through 25 days of expansion was promising in that the high and low-producing groups retained their difference in antibody productivity. The SLG system is able to detect and select cells as early as 2 days after plating, preventing fast-growing, but low-producing cells overtaking the population in each well. In addition, the process is simple, non-contact, and does not require any special washing or staining steps. The results indicate that the SLG method for selective cell growth in methylcellulose solution can be a viable alternative to current methods in optimizing antibody production.
6.2 Future Work

6.2.1 Improvement of Experimental Apparatus

Although the laser gelation system demonstrated feasibility, improvements to the experimental apparatus by decreasing gelation area and increasing throughput will improve overall performance. A specialized infrared focusing lens will minimize the focused beam waist and allow for a more controlled gelation area. However, using a specialized infrared lens will have poor imaging characteristics, so separate objectives must be used for imaging and laser gelation. The laser targeting will have to be implemented with great care will have to give accurate control of the laser positioning. Decreasing the time of laser scanning using galvanometric mirrors and path planning algorithms and improving the image processing pipeline will increase the throughput of the system. Improving the optics on the system would allow for a higher initial plating cell density and increased throughput.

6.2.2 Other Applications of SLG

While the laser gelation system was demonstrated by selecting high-producing antibody-secreting cells, this system can be generalized in other areas of cell culture with a mixed population of cells. This can be used to not just detect high-producing cells, but to screen cells for specificity prior to transfection by only changing the fluorescently-conjugated secondary antibody. The ability to detect and discriminate cells early in the culture process reduces the likelihood of a slow-growing cell line is overtaken by a fast-growing cell line. This can be useful for screening other cellular samples, such as microbial colonies.
References


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