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Microcarrier Separation Devices for Continuous In-Line Bioprocessing of Adherent Cell Culture

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MICROCARRIER SEPARATION DEVICES FOR CONTINUOUS IN-LINE BIOPROCESSING OF ADHERENT CELL CULTURE

by

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ABSTRACT

Bioreactors are a space-efficient method of growing cells en masse for industrial operations by suspending the cells in an agitated vessel full of cell culture media. Adherent cell lines can be grown on microcarriers in bioreactors to reduce the space needed for petri dishes or flasks in the lab. One of the important factors in cell culture is changing the cell media to remove cell waste, secreted products, and/or replenish the nutrients available. Bioreactors face unique challenges with media changes, as cells should not be removed from a culture when media is changed. Currently, some labs let the cells settle to the bottom of the bioreactor for 30-45 min before decanting the spent media and refilling the tank with new media. This limits the cells' access to oxygen and nutrients, and can cause the cells to die or stop producing a product of interest. This project provides bioreactor users with three single-use products that separate microcarriers from media in a continuous flow, to shorten the time adherent cells are outside of their ideal environment. The Honors extension of this project evaluates the adherent cell lines commonly used in industry, and calculates the stress induced on the microcarriers based on the fluid mechanics of the proposed designs. The designs selected for this project utilize gravitational and centrifugal settling with an inclined settler, lamella separator, and hydrocylone and were designed to operate with two peristaltic pumps to control flow rates, and separate a solution of dilute microcarriers. The Honors extension of this project also includes a robust explanation of the mechanism of settling for each design, and more clearly details how the results can be used to customize a separator to address the needs of a lab. Flow rates were tested to determine the most effective inlet and permeate streams to maximize settling for high flow rates. Two prototype designs – the inclined settler and the lamella separator – had a separation efficiency greater than 99% for a moderate flow rate above 2.083 L/hr (50 L over 24 hours), and

calculations of the maximum fluid shear forces were negligible compared to forces generated by bioreactor agitators. These designs can be scaled up to larger bioreactor cultures to allow for higher throughput and more efficient product separation, leading to longer bioreactor experiments with reduced risk of undue cell stress or death.

ACKNOWLEDGEMENTS

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INTRODUCTION

With technology capable of gene editing and mass pharmaceutical production, industries have devoted time and resources to cell culturing environments that maximize production from cells or viruses in culture. In labs and industries that require large-scale adherent cell culturing, current designs for mass culturing include flasks and petri dishes. These flasks and large-scale petri dishes take up precious lab space, and provide a challenge to change media efficiently and with little cell detachment. A solution to the problem of maximizing space is to suspend the cells in a liquid medium, which has resulted in variations of a device typically called a bioreactor. A bioreactor is a cylindrical vessel that can hold cells and liquid media and agitates the mixture so cells stay suspended in the media. These bioreactors also provide a closed environment that can be controlled to dictate the growth phase of the cells.

A bioreactor contains a variety of implements to avoid mass cell death and track cell growth[1]. The cell culture medium is stirred by impellers to keep the cells in suspension and inhibit cells adhering to each other. The medium is oxygenated with pure oxygen gas to allow for the cells to exchange oxygen by bubbling oxygen through the medium. Many bioreactors have other processes to limit cell death, such as: monitoring pH levels, foam levels, and the concentration of cells in solution. By monitoring pH levels, software in tandem with bioreactor technology can add in buffers to keep the medium at a consistent pH to avoid cell death. Foam level monitoring is used to prevent the bioreactor from overflowing with foam produced from bulk media interactions between the medium and dissolved gasses[2]. Cell concentration monitoring within a bioreactor provides control to ensure that cells are not leaving their ideal growth phase.

Mammalian cell culture produced a number of challenges for suspended culture, as many mammalian cell lines require an attachment to a surface in order to grow and proliferate. Industrial use of adherent cell bioreactor cultures includes the production of antibodies, vaccines, recombinant proteins, viruses, and in some special cases artificial organs[3]. A method of suspending adherent cells is to seed them onto microcarriers that are then suspended in the bioreactor. Microcarrier adherent cell cultures allow the growth of anchorage dependent cell lines as a single monolayer on the surface of the microcarriers, or as a multilayer embedded inside the microcarrier. Microcarrier cell cultures offer all the advantages of suspended cell culture to anchorage-dependent cells. Sinskey et al. has produced Sindbis viruses from both large scale microcarrier culture and individual roller bottles and has experienced a 50-fold increase of volumetric yield with the use of the bioreactor[4]. Park et al. had an improved gene expression of human embryonic stem cells on microcarriers compared to standard monolayer growth[5]. Many studies have demonstrated an increased yield in human mesenchymal stem cells, ashwagandha, and yeast compared to other adherent and agitated cell techniques, such as shake flasks[6–9]. The use of bioreactors and microcarriers also allows batches to be scaled up and down more easily than traditional 2D cultures, as seen in the study by Goh et al. with human fetal mesenchymal stem cells[10].

The Cytodex™ 1 microcarriers are a cross-linked dextran matrix for cells to attach to, which has a hydrophilic diethylaminoethyl-dextran exchanger and a positive charge on its surface. These properties allow for cells to preferentially attach to the microcarriers instead of the walls of a flask or bioreactor[11,12]. Dextran-based nanocarriers have also been proven to be an efficient way to reduce the amount of media used in standard bioreactors, which in part reduces the contamination risks; making them ideal for industrial processes[13].

However, microcarrier adherent-cell culture must be closely monitored to reduce the risk of cell detachment due to factors such as shear stress or cell death. If the cells are exposed to certain high shear conditions they may detach from the microcarriers and die[14]. Shear could be caused by hydraulic forces caused by impeller movement or the turbulent forces generated by the pumps used to move cells into the bioreactor. While shear stress will not always cause detachment in particles, the shear stress cells are subjected to could change their growth phase or metabolite production – thus limiting the quantity of product produced.

Advancements in the bioreactor field are continuous as new technology is developed to increase product yield and recovery. In 2017, Thermo Fisher Scientific presented their newest process to retain microcarriers from bioreactor batches in a single bag – named a Harvestainer™ BioProcess Container – to separate the microcarriers and adhered cells in a closed system[15]. This process allows for microcarriers and cells to be retained for reuse or product retrieval from supernatant by utilizing a filter mesh with nano-sized pores and has been successful in improving product yield in bioreactor use[16,17].

Bioreactor users harvesting viral particles, chemical products or patient cells from a batch of mammalian cells grown on microcarriers face the challenge of refreshing the culture media without damaging the cells. Current practices to remove spent media involve deactivating the bioreactor and allowing the microcarriers to settle for 30-45 min before decanting the media and adding new media[18]. No oxygen or nutrients are available to the cells in this time, which may result in cell death and product loss[18]. The goal of this project is to provide a method of separating the microcarriers from the spent media in a much shorter time frame, like 10 min, to reduce cell death, with simple, accessible, and scalable designs.

Cell Use in Industry [Honors Capstone Extension]

Cells on microcarriers suspended in an agitated bioreactor are far more likely to experience more severe hydrodynamic forces than non-adherent cells suspended in an agitated bioreactor[19]. The integrated shear factor – defined by Sinskey et al. as the laminar shear rate between the impeller tip and the walls – has been used to determine the death rate of cells within a stirred bioreactor[4]. However, as the size of bioreactors has increased over the decades, the integrated shear factor has been modified to better represent the forces that cells adhered to microcarriers experience while in a bioreactor[19].

As cells attach to the microcarriers, they go from their seeding morphology of round cells to a more spread out form as they attach themselves more securely to the surface of the microcarrier. The forces needed to shear the cells from microcarriers differs on the stage of attachment, but through numerical analysis 0.25 to 0.6 Nm^{-2} was found to be the range where round cells detach from the surface of a microcarrier[19]. The forces required to detach flattened attached cells from the surface of a microcarrier is dependent on the cell line attached to the microcarrier[19]. A study by Grein et al. suggests that the maximum shear stress levels for the Vero cell line are in the range of 3.5-5 Nm^{-2} for microcarrier-attached cells [20].

To ensure the controlled and steady growth of microcarrier-adhered cells, shear rates must be kept below the maximum tolerable shear for mammalian cells. The Cytodex 1 microcarrier manual gives a maximum velocity of 26.38 m/s, above which cells are more at risk to shear off of the microcarriers[21].

Centrifugal, Gravitational, and Other Settling Techniques

A defining factor in setting particulates out of a solution is the density of the particulates compared to the density of the solution, as particles of the same density of the fluid will not settle out of solution[22]. Another defining variable is the force acting on the particles to allow them to settle, as without any forces acting on the particles, their only method of movement is Brownian motion. Gravitational and centrifugal settling are commonly used in industry to settle particles out of solution[22]. On a large scale, gravitational settling requires less power, but often requires a larger space to allow for particles to settle out of a continuous flow[23]. Gravitational settling techniques are most efficient in static or laminar flow environments to allow for settling to occur without any agitation[24,25]. Centrifugal techniques are often most efficient in batch processes or with particles that have a significantly different density from the fluid they are suspended in[22].

The simplest method of separating microcarriers from a bioreactor involves ceasing all agitation and allowing for the cells to settle to the bottom of the bioreactor via gravity for however long that may take. Inclined settling tubes work similarly to the bioreactor, as they are a closed cylindrical device set at an incline. The feed solution – containing unseparated particles in solution – enters near the middle of the tube and as the solution fills the tube, the particles have the time to settle to the bottom and exit through a feed port at the bottom of the tube and the particle-free fluid exits through the top of the tube. Inclined settling tubes and chambers allow for high settling of the microcarriers, though most designs in industry utilize filters at the fluid outlet in their design to ensure maximum efficiency[26]. By inclining the settling tubes, convective currents within the system are minimized and the total distance required for particles to settle is reduced[26].

A modified version of the inclined settling design adds a barrier near the inlet source in the separator to drop the microcarriers out of fluid quicker, like a lamella separator. Lamella separators are primarily used in wastewater purification due to their compact design[27]. Lamella separators are modeled to have a series of inclined planes that the inlet stream will flow through[28,29]. As the water flows through the system, the laminar flow of the water allows for the solids to settle out of solution to the bottom of the device[30]. The clean water is taken off at the top of the device, and the solid waste is removed at the bottom of the device[31].

Hydrocyclones are an example of a continuous form of centrifugal settling, as they generate a centrifugal force dependent on the feed flow rate and the dimensions of the device. Hydrocyclones impart a centrifugal force to an in-flowing liquid to separate the light and heavy components of the solution[32–34]. Lighter components move towards the center of the hydrocyclone and up into the outflow port on the top of the device[35]. The heavy components are moved to the outer walls of the device and drop to the outflow port at the bottom of the device[35]. Hydrocyclones do not have as large a cost as other separation devices that impart a centrifugal force to the incoming liquid, as hydrocyclones manipulate the fluid at the inlet whereas other devices manipulate the environment that the solution is in[36]. Hydrocyclone separators are commonly used to remove sand and other solid particulates from water, where the difference in specific gravity of the particulates is larger than 1.5 SG[37]. The hydrocyclone has been modified and used to separate perfusion cells from media in a bioreactor before with success[25,38].

AIMS & OBJECTIVES

The overall aim of this project is to design and develop a product that will be used to separate microcarriers with adherent cell cultures from used media during a continuous flow process. The developed product will shorten the time cells are outside of their ideal environment during exchange of media in bioreactors, from approximately 45 min of cell settling to a goal of 10 min. The product will also be a single-use unit and the separation of cells on microcarriers from waste media will enable multiple uses of the same cells for more growth or production. For the design to be feasible, it must ensure that the microcarriers are not subjected to high shear stresses in fluid during separation. The design must be able to be scaled up to be compatible with industry scale bioreactor cultures for ideal use – from 50 L to 2000 L bioreactor capacity.

To achieve the aim of this project, the following objectives will be completed:

a. Dyes for microcarriers will be researched and tested to ensure accurate and simple calculations of microcarrier concentration
b. Multiple microcarrier settling designs will be reviewed and rated for feasibility prior to prototyping
c. The highest rated designs will be created using parts from local hardware stores
d. The initial prototypes will be tested to determine their feasibility and remodeled as necessary
e. Modified prototypes will be created using a 3D printer as necessary
f. Prototypes will be tested with a dilute microcarrier solution to determine efficiency of microcarrier separation versus the amount of solution separated

METHODS

Microcarrier Preparation, Dye, and Settling

The Cytodex™ 1 microcarriers utilized for this project were supplied by Thermo Fisher Scientific in a Harvestainer™ chamber. The Cytodex 1 microcarriers provided had been used in experiments for a urea broth solution, and were rinsed once with ddH_2O in the Harvestainer bag to remove excess urea. The microcarriers were dried in a vacuum oven at 215 °F for 4 hours to remove any excess water. After the microcarriers had been dried, they still retained some moisture, but were not saturated with water or urea.

As Cytodex 1 microcarriers are transparent when suspended in solution, it was necessary to dye the microcarriers in some way to visually track their movement within the settling devices for optimization. There are no standardized dyes to use with Cytodex 1 microcarriers, thus dyes were tested to assess their quantification potential. The dyes were judged on: absorption by microcarriers, amount of leaching into water when resuspended, availability, and visual observation. The three dye candidates were selected based on availability and ease of use: indigoidine, methylene blue, and rhodamine-B.

Once the microcarriers dried, the dye of interest were added to 0.5 g dried microcarriers and allowed to sit for 20 minutes. The indigoidine dye was dissolved in dimethyl sulfoxide (DMSO), and added to the microcarriers. After sitting for 20 min in the dye, the microcarriers were washed with ddH_2O to remove excess DMSO and unabsorbed dye. The microcarriers were then placed in a scintillation vial with 10 mL ddH_2O. Several dilutions from 1 g/20 mL to 1 g/20 L were prepared from the original concentration for visual analysis. A concentration of 5 g/L was used for all prototype testing. A protocol to dye the microcarriers using indigoidine was used for the remainder of the project (Appendix B).

Through initial prototype testing, the high flow rate required for efficient separation in the hydrocyclone resulted in the peristaltic pumps running above 200 RPM and shearing the microcarriers. All subsequent testing was completed at flow rates below this speed on the peristaltic pumps to avoid microcarrier shear.

An important factor of the gravitational settling process is the settling velocity of particles in a fluid, and how the density of a particle relates to the density of the fluid. The forces of gravity, drag, and buoyancy are the determining factors of the settling velocity of a particle in a fluid. By writing out the terms of each force calculation and rearranging the equation to solve for the velocity of the particle, we can determine the velocity at which a particle settles in our fluid. The settling velocity v_t (cm/min) can be determined using the general gravitational equation of terminal velocity of a particle in a fluid defined in Equation 1a[22]. The general centrifugal equation of terminal velocity of a particle in a fluid is defined in Equation 1b – substituting the force of gravity g with the centrifugal force $R\omega^2$, where R is the radius (m) and ω is the angular velocity (s^{-1})[22].

$$v_t = \frac{d^2(\rho_p - \rho_f)g}{18\mu}, \quad v_t = \frac{d^2(\rho_p - \rho_f)R\omega^2}{18\mu} \quad \text{(1a,b)}$$

Cytodex 1 microcarriers, have a specific density of 1.03 g/cm^3, compared to the specific density, 0.997 g/cm^3, of water[21]. Because the specific densities of the particle a fluid are relatively close, the settling velocity still exists but the gravitational settling method is not incredibly efficient. However for this application, the negligible forces acting on the microcarriers ensures that cells on the surface will not be sheared off as they move and settle in a solution. The settling velocity of the Cytodex 1 microcarriers was determined experimentally by the manufacturers to be 12-16 cm/min[21]. This settling rate is affected by the dimensions of the vessel the microcarriers are settling in, namely the height of the vessel so it is a long wait time

for microcarriers to settle in industrial scale bioreactors. As viscosity μ (Pa·s) is also a factor in determining the settling velocity of a particle, the solution of dilute microcarriers was modified to more accurately represent cell culture media. Through testing we found that a salt and pluronic (F68) solution provided the best analogue for a media mix.

Prototype Design and Fabrication

Five initial prototypes were proposed based on various methods of solids separation in a variety of industrial applications. A design matrix was developed to aid in determining the prototypes that were most feasible and fulfilled the requirements of the settling device (Appendix A, Table 1). Each prototype was researched, analyzed, and scored to narrow focus to three prototypes to be fabricated and tested. The table includes all designs that were researched: helical tube, inclined settler, modified inclined settler, cyclone settler and venturi separator. The table was weighted based on the importance of each criteria assigned by the Thermo Fisher employees from 1 to 10 – 10 being the most important – the weight of the criteria is calculated off of those numbers. The inclined settler had the highest score of the matrix, with the modified inclined settler (later renamed Lamella separator) and hydrocyclone separator following closely behind. The venturi and helical separators were scored poorly due mainly to the forces exerted on the microcarriers as they are separated from the system. Thus, we began fabrication of prototypes for the inclined settler, lamella separator, and hydrocyclone separators.

Once fabricated, the prototypes were tested using a written standard protocol to retain accuracy in testing between the individual prototypes. The comparisons between prototypes fall to the differences in efficiency of separation and the permeate flow rate used. Thus, the same testing protocol was followed for testing each prototype (Appendix B).

Upon testing the prototypes we found that the microcarriers stuck to the surface of the inclined settler and lamella separator prototypes, and a range of tests were completed to make the surface of the prototypes more hydrophobic, decrease surface interactions, and change the ionic charge of the microcarriers. To increase the hydrophobicity of the surface of the prototypes we applied RainX – a commercially available spray that increases the hydrophobicity of a surface – to reduce the adhesion. After the applying RainX, we observed the microcarriers sticking to the surface of the prototypes less (and releasing from the surface completely when flushed with water), but they were still adhering to the surface of the prototypes during the settling experiments. We tested different combinations of pluronic F68 and NaCl in the dilute microcarrier solution to change the surface charge of the microcarriers and the prototype materials. The pluronic F68 is commonly used in bioreactor cell culture to protect cells from hydrodynamic and bubble-induced shear within the system[39]. After some testing, we settled on a solution of 1 g/L NaCl to provide an ionic buffer to help prevent the microcarriers from sticking to the prototype – and subsequently used that solution to conduct the comparative efficiency experiments.

Hydrocyclone

The hydrocyclone prototype was designed using the Bradley hydrocyclone[40] (Appendix A, Hydrocyclone Design #1). Materials were purchased from Home Depot to build a rough prototype for a hydrocyclone separator. The flowrate for proper separation was higher than recommended for the microcarriers used, resulting in microcarriers breaking due to stresses from the peristaltic pump during initial tests. The research by Ahmed provides a design for a hydrocyclone that separates cells from suspended culture[41]. A value for the underflow diameter was given, 2.5 mm, and by calculating backwards the dimensional ratios of the hydrocyclone

were identified (Appendix A, Hydrocyclone Design #2). The design was modeled in SolidWorks and sent to Thermo Fisher for 3D printing with an Objet 260 Connex 3 printer.

An optimal inlet flow rate was calculated to be 90 mL/min and a variety of return flow rates were researched to find the optimum separation. Multiple tests were completed at each percentage of return flow rate to ensure repeatability and consistent operation of device.

Inclined Settler

As there is no consensus in inclined settler dimensions in literature, previous experience with fluid mechanics decided the dimensions and locations of the inlet and outlet ports[42]. Since pump pressure is the driving force of the system, the size of the outlets is negligible. Control of flow was restricted by the pumps attached to the settler. The inclined settler prototype was assembled using materials purchased at Home Depot and tubing provided by Thermo Fisher. The main body of the prototype was clear PVC tubing cut to just over 2 ft in length to increase the vertical distance between the inlet and two outlets to facilitate higher efficiency. The inlet stream was placed $^1/_3$ of the length above the return outlet and $^2/_3$ of the length below the harvest outlet. Inlet and outlet ports had a diameter of $^1/_4$ in. The bottom of the PVC tube was sealed shut with a plug and silicone glue to prevent leaks, the top was left open for ease of cleaning.

The device was tested to quantify optimal inflow and outflow rates along with angles of incline for greatest settling efficiency. After initial tests, the inclined settler was modified to prevent leaks and degradation of the structure of the prototype with UV-cured silicone glue. Testing of the efficiency of the prototype was done by varying the inlet and outlet flow rates. Initial testing was done to confirm and industry incline of 55° to 60° as the optimal choice[43]. The inclined settler was tested at control flow rate at a 60° incline – 30° decline from a vertical line. Tests for 65° and 70° inclines were also completed to best optimize the settling effect.

Control angles of 90° and 75° were tested, to ensure a comprehensive review of optimal angle of operation. Flow rates were decided based on calculations done to determine the pressure differentials (Appendix A, Inclined Settler).

Lamella Separator

The lamella separator prototype was made from acrylic and silicone adhesive that had been purchased at Home Depot. Calculations were finalized to maximize the acrylic available and incorporate six 60˚ incline planes (Appendix A, Lamella Separator).

When the prototype was fully constructed, the volume was calculated and verified to be approximately 3 L.

The device was tested to investigate inflow and outflow rates that would result in the highest microcarrier separation efficiency. Initial tests were completed, and a flow rate of 96 mL/min was found to successfully separate microcarriers at the desired efficiency. Initial testing consisted of collecting the microcarriers at the bottom of the device while only having an inlet flow and permeate flow – both at 96 mL/min.

In order to test increased inlet flow rates, the rubber stopper was removed from the bottom of the vessel and an outlet for a return flow was added. Tests were run to determine the maximum inlet flow rate that could be achieved while maintaining a high microcarrier separation efficiency. Inlet flow rates up to 350 mL/min were tested and the device was found to successfully separate the microcarriers with a high separation efficiency.

Throughout the duration of testing, the edges and seams of the lamella separator were re-caulked, as leaks continued to appear throughout all testing experiments. Testing on this device was discontinued before the maximum inlet flow rate was found, as cracks in the device rendered the device unusable.

Later, the prototype was designed in SolidWorks to allow for more optimization of the design. This approach promoted a more economically and environmentally sustainable design and fabrication process. The SolidWorks file of the design was 3D printed by Thermo Fisher Scientific to reduce dead space, make the design more compact, and prevent leaking. The device was printed using RGD720 material, printed with the Objet 260 Connex 3 printer.

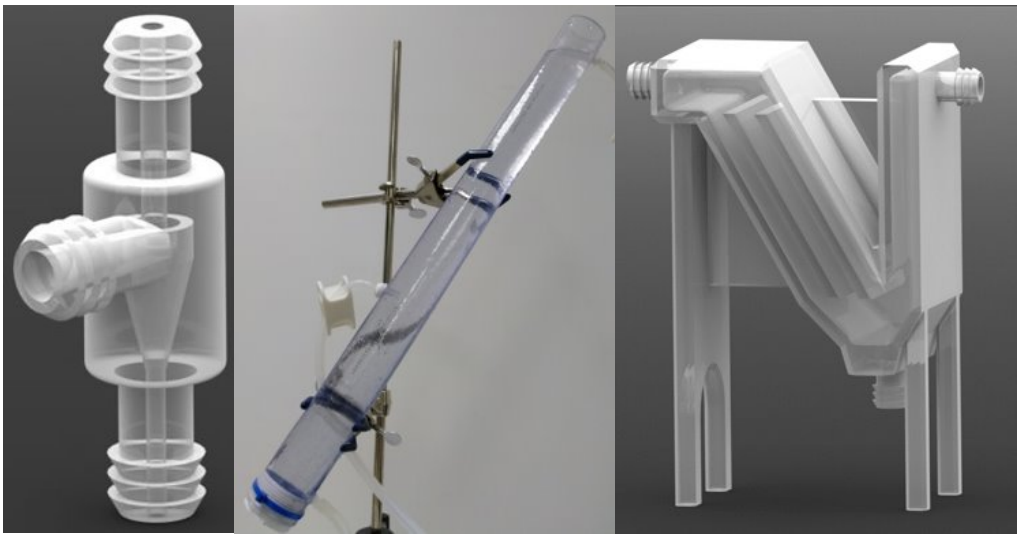

Figure 1. The redesigned SolidWorks model of the hydrocyclone separator, a picture of inclined settler, and the SolidWorks model of the redesigned lamella settling device. (left) Second hydrocyclone design modeled in SolidWorks. All dimensions taken from preliminary prototype were scaled down to millimeter scale, and inlets and outlets were modelled to have a diameter of ¼ in. (center) Inclined settler prototype, pictured during settling experiment, the degree of incline controlled with test tube clamps and a stand. (right) Second lamella separator modelled in SolidWorks, modifications included a total volume reduction and inlet and outlet ports modified to a ¼ in diameter.

Prototype Animations [Honors Capstone Extension]

Another extension to this project in improving the comprehension of this project was to create digital animations of the settling process of each prototype. Ideally, these animations would have provided a visual representation of the shear forces acting on the microcarriers for each prototype design, and would be more easily accessible for a larger audience than calculated shear stress numbers. Unfortunately, due to the unexpected circumstances of Spring 2020, the animations could not be fully developed, and thus the project had to continue with a more

comprehensive explanation of the mechanism of separation for each prototype, and theoretical cell shear analysis for the gravitational settlers.

Cell Shear Analysis [Honors Capstone Extension]

Cell shear analysis for the devices was completed by calculating the Reynold's number for the highest flow rate for any device that had above 99% efficiency. The Reynold's number was used to determine if the flow into the system was laminar or turbulent. The effect of wall shear stress and orifice friction generated in the system was calculated.

RESULTS AND DISCUSSION

Microcarrier Dye

The methylene blue dye was quick to leach out of the microcarriers, and after a wash with ddH_2O they did not have any noticeable coloration. Upon further research, we found that methylene blue dye specifically binds to ferrous molecules in biological proteins[44]. As the microcarriers have dextran-based surfaces, there is no ferrous molecule for the dye to bind to (Figure 2). The rhodamine-B dye leached out of the microcarriers after 5 min of sitting in the water. The rhodamine-B dyed microcarriers also agglomerated and floated to the top of the water while sitting in ddH_2O (Figure 2). After more research, rhodamine-B was found to be acidic which was causing the microcarriers to dissolve[45] (Figure 2). The indigoidine dyed microcarriers were clearly visible to the naked eye, and could be captured on camera to observe the microcarriers settling over time (Figure 3). Indigoidine was selected as the dye of choice for visual tracking of the microcarriers in solution with the success of dyeing of the microcarriers.

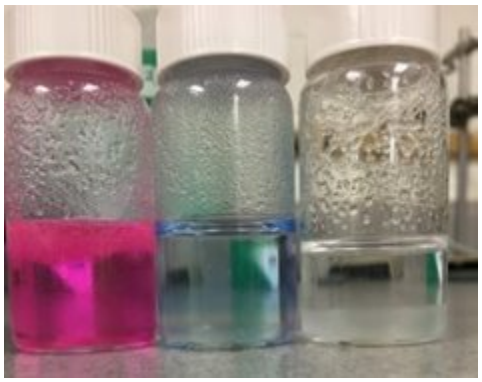

Figure 2. Rhodamine-B dyed microcarriers, methylene blue dyed microcarriers, and undyed microcarriers suspended in ddH_2O. (left) Rhodamine-B has a solution that is a red color, indicating that the dye has leached out of the microcarriers, and microcarriers are floating at the top of the solution. (middle) Methylene blue has no noticeable microcarriers in solution and the solution appears to be dyed blue. (right) In the undyed microcarrier solution, there is no visible evidence of the microcarriers in solution other than an more opaque band near the base of the vial, but microcarriers are observed sticking to the sides of the vial.

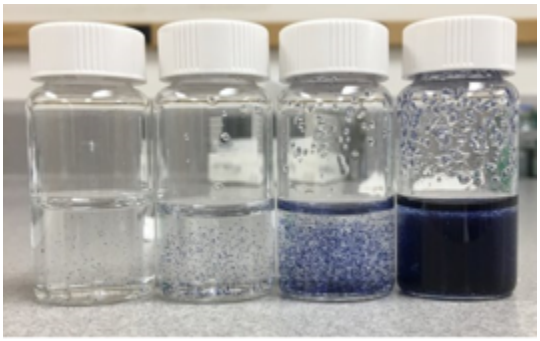

Figure 3. Indigoidine dyed microcarriers suspended in ddH_2O at a series of dilutions to demonstrate distinct visual differences in microcarrier concentration. The dilutions are as follows (left to right): 1:10,000, 1:1,000, 1:100, and 1:10 g/mL in ddH_2O. Even at a 1:10,000 g/mL there are visually noticeable dyed microcarriers in solution, and at 1:100 g/mL dilution there is a noticeable settling pattern observed as the microcarriers settle.

Microcarrier Solution Testing

Upon further research of visual and digital methods of tracking dyed microcarrier concentrations in solution, the method of quantifying microcarriers in solution was changed to improve efficiency in collecting data. All comparative microcarriers separation data was collected by filtering microcarriers from the harvest and permeate streams with micron mesh and weighing them with a mass balance. This was done with undyed microcarriers largely to avoid any mass discrepancies because of the added mass of absorbed dye.

Efficiency Testing

As efficiency testing progressed, combinations of pluronic F68 and salt were used to reduce microcarrier adhesion to the surface of the inclined settler and lamella separator prototypes. The two designs experiencing the microcarrier adhesions were the two that use gravitational settling to separate microcarriers. This use of gravity to separate the microcarriers allows the microcarriers more contact with the surface of the prototype, as they are moving slowly through the system. Though we were unable to do a further study on the reasons for this adhesion due to time constraints, we postulate that the substantial contact between the microcarriers and surface of the gravitational settlers combined with the positively charged surface of the dextran on the microcarriers resulted in surface charge interactions between the dextran and the uneven static charges along the surface of the PVC and resin used for these settling devices[46]. The adhesion resulting from the interactions was decreased if the inlet flow was increased for these prototypes, but the resulting turbulent streams would disrupt the microcarriers and not allow them to settle efficiently. The pluronic F68 and salt solution used to simulate a cell culture media solution helped to reduce this adhesion, but did not eliminate it.

The efficiency of each separator was evaluated based on the ratio of mass of microcarriers collected in the permeate in relation to the total mass of microcarriers introduced to the system, and the rate of return flow. The aim of the project was to achieve a microcarrier mass separation efficiency of above 99% and a rate of return flow at or above 50 L per day (24 hours).

Hydrocyclone Separator

The hydrocyclone separator faced unique challenges in separating microcarriers because it utilizes centrifugal forces as a mechanism separation. The premise of the centrifugal separation used in the device is that as the dilute microcarrier solution enters the inlet and flows along the

cone interior to form a vortex[47]. As the solution is flowing, heavier particles move to the outside of the vessel due to the centrifugal force of the solution moving in the vortex and the less dense particles flow to the center of the device, forming an inner vortex[48] (Figure 4a). Water flows around the surface of the cone but is not pushed completely to the walls of the cone[48]. When the water reaches a specific diameter of the vortex near the bottom of the cone, it moves through the low pressure center of the device creating an inner vortex that flows upwards and out the top of the device[48] (Figure 4b). Not all water moves through the top of the device, some is retained with the microcarriers and falls out the bottom of the device, but theoretically a majority of the water would leave the system at the top of the device[43]. The microcarriers enter the conical chamber of the hydrocyclone and are pushed to the outside of the outer vortex because of the centrifugal force of the solution moving along the cone shape[48]. The microcarriers then travel to the bottom of the hydrocyclone, but because of their migration towards the walls of the cone they drop from the bottom of the device into an outlet stream[43] (Figure 4c).

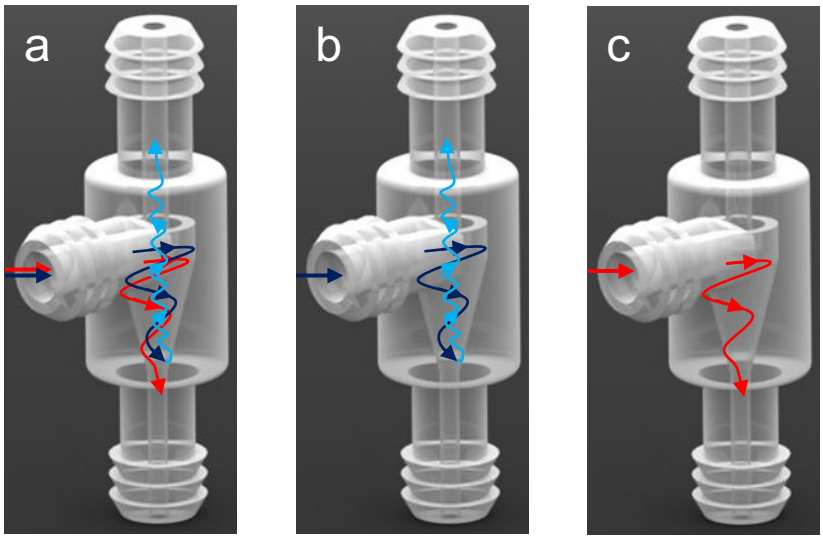

Figure 4. Diagram of hydrocyclone separation mechanism. The SolidWorks model of the hydrocyclone is shown with a) the entirety of the separation mechanic in a series of flow paths – entering fluid flow in dark blue, exiting fluid flow in light blue, and the entire particle flow in red; b) the movement of fluid through the cone, the dark blue path indicates initial entrance and flow behavior, the light blue path indicates the inner vortex creation at an arbitrary vortex diameter and flow in the inner vortex through the top exit of device, c) the red path indicates the particle stream as particles move through the outer vortex and drop through the bottom of the device.

The hydrocyclone functions best when the inlet stream has a high flow rate, because the centrifugal force is dependent on the inlet flow – the faster the flow into the system, the more centrifugal force the fluid undergoes, the more particles can be settled out of solution. The high flow rates required high RPM from the peristaltic pumps, which destroyed the microcarriers. Further research will be necessary to find better dimensional ratios and scale to provide greater time for separation. Future iterations of the prototype could improve efficiency by increasing the length of the conical separation section. Increasing the conical separation section would increase the time the microcarrier solution is experiencing centrifugal force. With more exposure to centrifugal force as they travel through the system, they would be closer to the walls of the device and thus farther from the inner vortex. Because of the increased distance between the microcarriers and the inner vortex, there would be less microcarriers getting caught in the inner vortex, and thus improve the efficiency of the design. Increasing the conical section, would allow us to run the hydrocyclone at 90 mL/min, but nonetheless increase the separation of the microcarriers from solution.

The testing of the hydrocyclone was completed at an inlet flow rate of 90 mL/min – as that was the highest inlet flow rate that did not shear the microcarriers – and the goal was to find the return flow percentage that would provide the greatest efficiency of microcarrier separation.

Initial tests were executed for 33%, 60%, and 90% return flows and evaluated qualitatively to find the return flow range that would yield the best efficiency. The 90% flow had the greatest return flow efficiency so testing was done above and below 90% return to identify the most efficient flow rate. We tested return flow rates of 75%, 80%, 85%, 90%, and 94% to identify a “breaking point” or drop in efficiency in the lower range of return percentage. The greatest efficiency occurred when Using a return flow of 85% gave the best efficiency, at an

average of 86% separation efficiency, with a maximum of 91% efficient microcarrier separation. Using 90 ml/min and a return flow of 85%, the hydrocyclone would be able to separate a total of 19.44 L per day. This total is well below our aim of 50 L per day for a single device, but if run in parallel it would take three hydrocyclone designs to separate more than 50 L per day.

The results show that the current prototype scale and the operating parameters used are insufficient to produce greater than 99% efficient separation, as well as unable to meet a separation rate of 50 L over 24 hrs (Figure 5). The 90 mL/min inlet flow rate produced a consistent percent separation across several return rates, which reflects literature values for a hydrocyclone separation efficiency of ~70-80% in industry[38]. When the outlet flow rate of the hydrocyclone is graphed against the efficiency of separation, we see a weak correlation between flow rate and efficiency. No linear, power, exponential, logarithmic or high order polynomial trendline gives an accurate assessment of the data presented. This is due to the variance in efficiency between different tests of the same return flow rate percentage, and a lack of trend in efficiency as the return rate changes. With the polynomial trendline generating the highest R^2 value of 0.2729 – a weak correlation between the data and the trendline – we can conclude that this data does not have any trend and cannot be extrapolated from to predict the efficiency of the device at higher return flow rates (Figure 5).

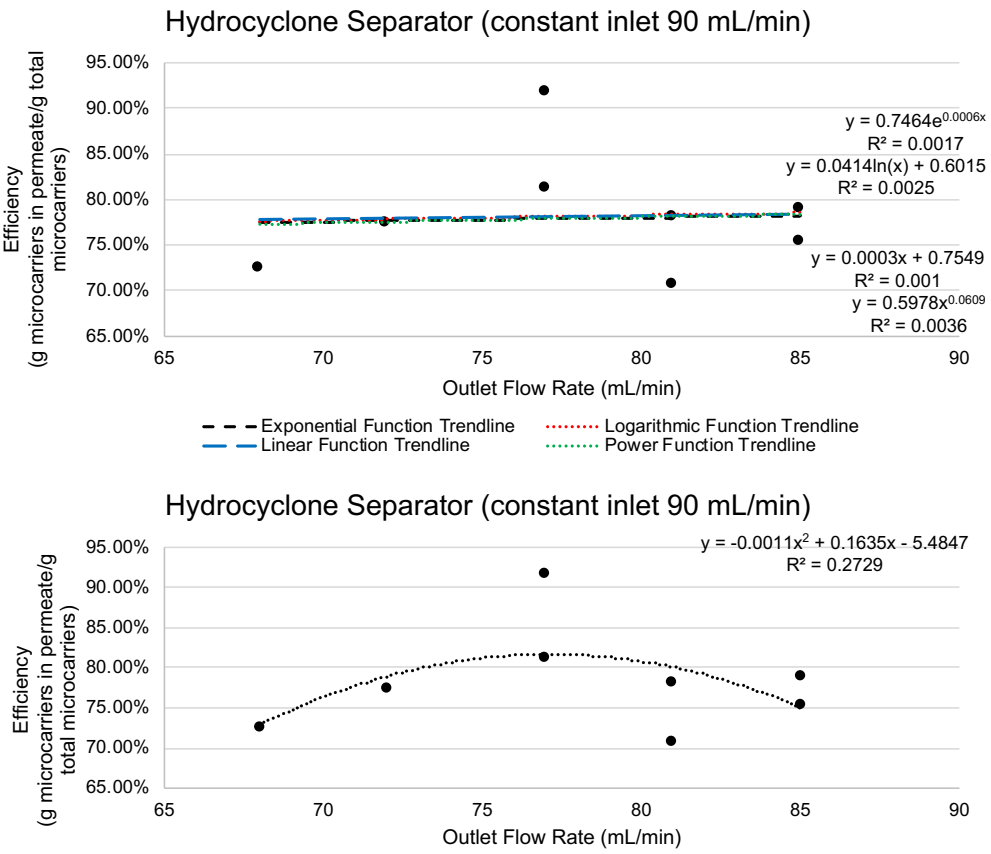

Figure 5. Graph of hydrocyclone efficiency of separating microcarriers at an inlet flow of 90 mL/min over different outlet flow rates. No trendline fits the data at an acceptable R^2 value of >0.95 to conclude that any trendline is indicative of a graphical trend. The outlying data points result in inaccurate models of efficiency in relation to the outlet flow rate. The polynomial function trendline (bottom) becomes more accurate with a higher order polynomial fit, but does not give accurate interpolative or extrapolative behavior.

Inclined Settler

The testing of the control angles of 90° and 45° showed efficiency under 70% but the literature value of 60° provided the greatest efficiency above 90% for all tests[26]. Thus a 60° incline was used for the inclined settler throughout testing to optimize efficiency.

Various inlet and outlet flow ratios were then tested to verify the conditions for highest separation efficiency. The highest efficiency was obtained at a flow of 60 mL/min inlet and a 33% return flow, resulting in a predicted total of 57.6 L of potential permeate obtained in 24 hours. The breaking point where the lowest efficiency was obtained was found at 150 mL/min at 25% return flow, which had a predicted total of 162 L of permeate collected. The efficiency of

the inclined settler dropped as inlet rate increased, however the capacity of the inclined settler is ideal to scale up the operation without changing the design of the device.

Two tests were performed at each condition for 33% return ratio, and once at each condition for 25% and 40% return. The greatest stability and efficiency of operation was found at a 33% ratio. The 25% ratio removed too little microcarriers from the product and resulted in lower efficiency. The 40% efficiency was efficient at removing the microcarriers, but collected the least permeate overall.

The results show that the current scale of the prototype is able to produce the desired efficiency for the operation of a 50 L bioreactor, but decreases in efficiency for higher flow rates. The R^2 value shows that the data trend follows a strong correlation of 0.9693 for the 40% return rate, which can be used to interpolate the efficiency of the prototype at a 40% return rate for other inlet flow rates (Figure 6).

Through the experimentation process we found that the inclined settler took up more lab space than anticipated and there was a noticeable adhesion of microcarriers to the inside surface of the tube. Additionally, there were settled microcarriers that were unable to leave the device around the glued ports, as the extension of the port into the tube created a dead space at the return flow port.

Future experimentation would include chaining prototypes in series to reduce the lab space used to run the inclined settler design at a larger scale. The efficiency of this prototype can be increased with a more hydrophobic or positively charged material to reduce interactions between the microcarriers and the prototype surface. Future iterations would include ports that are integrated into the structure of the prototype to reduce pockets of settled microcarriers that are unaffected by the flows within the prototype.

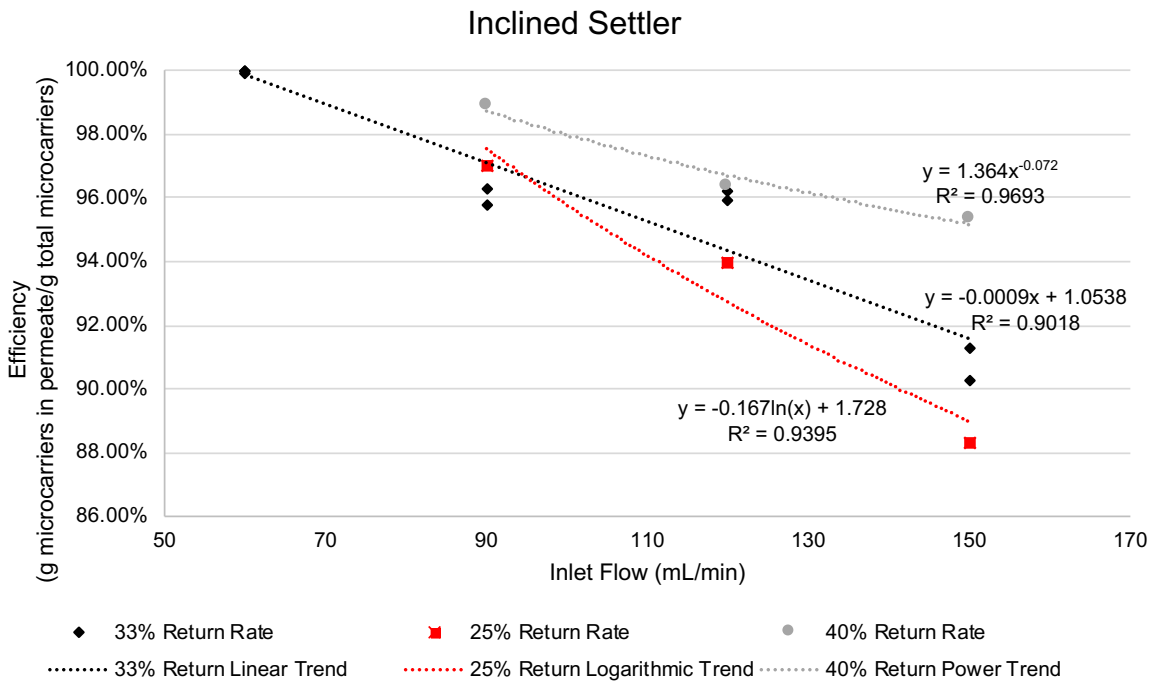

Figure 6. Graph of the efficiency of separating microcarriers with the inclined settler device over inlet flow rates at different return rate percentages. As the inlet flow rate increased, overall the efficiency of the inclined settler decreased. The trendlines generated by the obtained data can be used to interpolate and potentially extrapolate with error for the efficiency of other related flow rates because of their relatively high R^2 values indicating correlation between the equation and the behavior of the data.

Lamella Separator

To test the separation efficiency of the lamella separator, ten tests were performed at a variety of inlet and outlet flow rates to identify high and low efficiency conditions – some with less duplicates than others due to time constraints. An inlet flow rate of 75.6 mL/min with a 60% return flow resulted in the highest efficiency, and results in a permeate volume of approximately 43.5 L over the course of a day.

We tested a series of conditions determine flow rates that would satisfy our 50 L per day aim while maintaining a greater than 99% efficiency. A inlet flow rate of 76 mL/min and a 33% return flow had >99% efficiency and processed approximately 73.0 L of permeate in 24 hours, which is 20 L above the aim for this project of 50 L permeate processed per day.

The prototype scale of the lamella separator is capable of separating microcarriers from solution at a >99% efficiency for the operation of a 50 L bioreactor in the course of a day. The data collected for the 33% return rate generated a polynomial trendline that had a high R^2 value of 0.9973, indicating a high correlation between the behavior of the data and the trendline (Figure 7). This trendline could be used to find that the maximum inlet flow that can be achieved while maintaining a separation efficiency of at least 99% for a 33% return rate.

This design is practical for lab usage as its footprint only requires an area of 60 cm^2 with a height of 13.5 cm. This design could be easily scaled-up by increasing the internal volume and dimensions, thus allowing more feed to be processed by the single device, or by chaining the prototype in series to process more feed at once. The lamella separator is a flexible microcarrier separator because of the wide range of inlet flow and return rate combinations that have are >99% efficient give users the ability to modify the lamella separator to fit their needs.

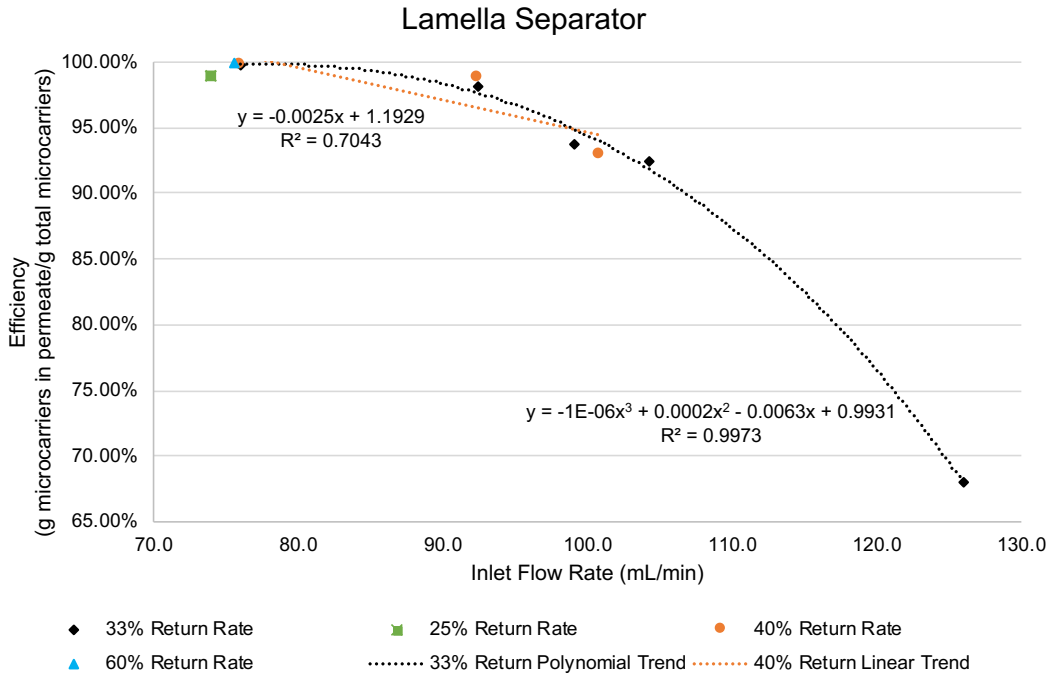

Figure 7. Graph of efficiency of microcarrier separation using the lamella separation device over varying inlet flow rates at different return rate percentages. The markers indicate different return rate percentages. As a general trend the efficiency of separation decreases for all return rates as the inlet flow rate increases for the system.

Cell Shear Analysis [Honors Capstone Extension]

To ensure the controlled and steady growth of microcarrier-adhered cells, shear rates must be kept below the maximum tolerable shear for mammalian cells. The Cytodex 1 microcarrier manual gives a maximum velocity for mammalian cells of at 26.38 m/s, else the cells are more at risk to shear off of the microcarriers[49]. When the microcarriers travel through a $^1/_6$ in diameter tube at 90 mL/min, they are travelling at equivalent velocity of 0.107 m/s – which is significantly below the threshold for adherent cell tolerable shear.

The overall effect of gravitational microcarrier separation of the inclined settler and lamella separators is not enough to cause a change in growth phase or detachment. The flow of the microcarrier solution at 90 mL/min is laminar through a $^1/_4$ in tube, and thus there is a negligible shear effect of the microcarriers going through an orifice or moving with the fluid (Appendix A, Cell Shear). The wall shear stress of the fluid flowing through the tube is $-2.149 \cdot 10^{-4}$ Pa and since the microcarriers are only in contact with the wall of the tube for a small amount of time as they move through the tubing with laminar flow, they experience a fraction of the wall shear stress calculated (Appendix A, Cell Shear). From these two calculations we conclude that the cells are not experiencing enough stress during transport to the settling devices and settling to detach or change growth phase. In fact, cells likely experience more shear forces from the impeller movement inside a bioreactor, as the computational fluid dynamic models of novel and traditional impeller devices ranges from 0.2-1 Pa[14,50,51].

The forces generated by the hydrocylone intersect at centrifugal and fluid shear forces, due to the limited success of the hydrocyclone at the 90 mL/min flow rate limit we worked at – calculations are impossible without replicable fluid behavior and microcarrier separation within the hydrocyclone chamber. However, based on preliminary tests, we observed that the shear

forces generated by the pumps to adequately feed the hydrocyclone are enough to damage the microcarriers. With that knowledge, it follows that any contact of cells attached to microcarriers on the tubing or hydrocyclone itself at such speeds would generate sufficient wall shear stress to change cell growth phase or detach the cells. As this method of separating the microcarriers from a fluid is based in the generation of centrifugal force from vortex-shaped velocity, we assumed that the design we tested will require further refinement to take into account the forces acting on cells.

Scalability

The prototypes were scaled and operated for flow rates associated with the media exchange for a 50 L bioreactor in the course of a day. Flow rates other than 90 mL/min will need to be tested for the highest efficiency of the current hydrocyclone scale – as optimal conditions only generated 19.4 L of permeate per day. Further scaling up of this prototype will involve increasing the total size, as well as increasing the length of the conical cyclone section of the prototype. The inclined settler can be scaled up either by increasing the total length of separation (by increasing the tube size past the 2 ft of the prototype), or by incorporating multiple devices in series. The greater length or greater distance the microcarriers travel will allow for larger, more efficient flow rates for larger-scale bioreactors. The lamella separator can be scaled up with an increase in internal volume by increasing the dimensions of the body of the device. A greater number of thinner lamella sheets will facilitate greater reduction in convective currents through the lamella section, which should increase settling and efficiency to separate a larger volume of media. Overall, the plausible scalability of the prototype separator devices provides the project with potential to be easily scaled to larger bioreactor systems.

Scaling the devices up would have an insignificant impact on the shear forces experienced by cells adhered to microcarriers for the inclined settler and lamella devices. As the inclined settler and lamella devices rely on gravitational settling to separate microcarriers, they require laminar flow for the system regardless of scale – a scale up would not introduce any additional turbulence or shear forces into the system. The largest source of shear for these devices would be from the pumps used to transport the microcarrier solution to the device, if they exceed the upper limit of 26.38 m/s suggested by the manufacturer[21]. This would no doubt cause the shearing of the microcarriers and thus the cells off of the microcarriers. Scaling the hydrocyclone to larger sizes poses a large hurdle, as increasing the flow rate with this pump and tubing setup would most likely shear the microcarriers and cells irreparably.

CONCLUSIONS AND FUTURE WORK

Conclusions

This project culminated in the report above and three physical prototypes that we tested and gathered data for. We obtained used microcarriers, developed a protocol to clean them and successfully dyed microcarriers to aid in visual identification of settling. The indigoidine dye we selected for testing did not change the physical properties of the microcarriers, and bound to the dextran surface of the microcarrier without leaching. Though we were unable to use the dye to accurately quantify the microcarriers in solution, we instead weighed the microcarriers collected in the two outlet streams and determined the efficiency of the prototype. The ratio of the mass of microcarriers in the return feed versus the total mass of microcarriers collected was used to determine the settling efficiency of each device.

We researched five microcarrier settling devices and determined three to physically design and build based on criteria weighted by our Thermo Fisher partners. The criteria focused on the feasibility of fabricating the design and predicted efficiency of separating microcarriers from solution; as well as, sterilization potential and low shear conditions to limit cell death. The inclined settler, lamella separator, and hydrocyclone designs were rated highly for their high feasibility in efficiently separating microcarriers and simple designs. We created physical prototypes of each design out of hardware store materials that could be run with the peristaltic pumps we had access to. Upon preliminary testing, all designs successfully separated microcarriers from water, but still needed modification and refinement to achieve >99% separation efficiency at larger inlet flow rates. We addressed fabrication flaws, such as leaks or incorrectly sized connection ports, and redesigned the lamella and hydrocyclone designs. We redesigned the lamella separator and hydrocyclone separator to decrease leakage and dead space then used a 3D printer to fabricate more accurate prototypes.

Subsequently, we completed optimization testing to determine the conditions that would result in the highest efficiency and highest return flow rate.

The second design of the hydrocyclone separator was unable to exceed 95% efficiency on any one experiment, and thus unable to achieve the aim of filtering 50 L of permeate in a day at an efficiency greater than 99%. The optimal conditions of hydrocyclone operation could process 19.44 L of permeate in a day with 86% efficiency. The data we collected was widely varied with no perceivable relationship between permeate flow rate and efficiency.

The inclined settler performed best at an angle of 60° out of angles tested between 90° and 45°, validating the angles reported in literature and industry. At a permeate flow rate of 40 mL/min the inclined settler had a greater than 99% efficiency, and a permeate process flow

equivalent to 57.6 L processed in 24 hours. The inclined settler was able to achieve the for the processing rate for this project and the data collected showed efficiency generally decreasing as the flow rate of the system increased.

The second design of the lamella separator was separate a permeate flow rate of 50.7 mL/min at an efficiency greater than 99%, which would be equivalent to 73 L processed in a day. The design is compact and has a wide range of conditions to control the rate of permeate processed above 99% efficiency – making it suitable for lab benchtop and industrial bioreactor use. The data collected from the lamella separator follows a general trend of efficiency decreasing as the system flow rate increases, however it takes a dramatic increase of inlet flow to reduce the efficiency below 90%.

The forces acting on the microcarriers for the inclined settler and lamella separator were calculated and are theoretically too low to have a significant effect on cell attachment or growth phase. However, the forces generated by the microcarriers travelling through the hydrocyclone are certainly high enough to shear cells from the surface of a microcarrier, if not shear the microcarrier itself.

Overall, this project successfully produced two device designs that gravitationally settle and separate microcarriers out of solution at a rate of 50 L per day. These devices can be fabricated with inexpensive materials, and have the potential to be sterilized using autoclave or alcohol washes for cell culture. They can be used in research and development in industry to extend the length of a run, and minimize the effort needed to perform daily media changes.

Future Work

Future work will consist primarily of further testing of microcarrier settling efficiencies and new iterations of prototypes. Further research into hydrocyclone dimensional ratios and design iterations need to be explored for greater separation efficiencies.

The feasibility of scaling up the designs can be determined through larger scale designs and experimental testing to calculate a scaling factor to develop devices for any size of bioreactor. Additionally, scalability has potential for improvement by setting up a series of prototypes in parallel.

Cell shearing was concern in prototype design, and a method of quantitatively assessing cell shear from microcarriers would be beneficial to design prototype experiments and quantify the upper limits for cell shearing with each device. Each device can be tested with cell culture media and microcarrier-adhered cells to determine the differences between cell-microcarrier settling and non-cell microcarrier settling as well as any cell detachment or growth phase changes that result from the settling system. Alternative prototype materials will need to be tested for surface properties favorable for preventing microcarrier or cell adhesion to increase performance efficiency. Centrifugal pumps could be used as an alternative to peristaltic pumps, to reduce the microcarrier destruction we saw with peristaltic pumps. With refinement and further testing, all designs could be modified and tested to ensure that the forces cell-laden microcarriers experience in the separating devices do not cause significant cell detachment or growth phase change.

REFLECTIVE WRITING

The Honors capstone presented above is a continuation of the engineering capstone I submitted with a group of my peers this past semester to fulfill our major requirements. I chose to continue this project because it gave me the opportunity to research topics I am interested in – and the problem we were addressing directly involved cell function and revolved around the use of cells on microcarriers.

I knew that the research required to assess effects on cell attachment would be substantial and would not fit into the timeline for our major capstone. By extending this project for my Honors capstone, I was not only able to complete a more comprehensive review of the devices we designed, but I was also able to pursue research in topic I am passionate about. A comprehensive cell culture analysis of our designs was difficult to accomplish in one semester, so I focused my research on the known effects of bioreactor use on microcarrier-adherent cells. I took the lead on managing my team during our engineering capstone project and compiling the majority of the final report to be submitted. As team lead, I ensured that each team member was weighing in on decisions about where the project was going, everyone had the support to complete their goals each week, meeting notes were recorded, questions were answered, and that we were on schedule to complete each phase of the project on time. To compile the report, I completed an extensive literature review of patents, academic journal articles, textbooks, and other documents to assemble a robust foundation for explaining what a bioreactor is and the development of each device design from concept to calculation to fabrication.

This project was proposed with an animation of microcarriers settling in each of the devices developed by my team; however, due to campus shutdowns and the switch to remote classes – I was unable to complete this portion of my report. To compensate for that visual, I

endeavored to explain more of the general behavior of the microcarriers in each device in the report and completed fluid mechanics calculations to determine theoretical shear forces acting on the microcarriers as they move through the system.

I am incredibly interested in cell culture and the extension of this capstone to assess the effects of our devices on cells in culture gave me the opportunity to pursue literary research for bioreactor cell culture – which I thought I would never get to study because my lab focuses on adherent 2D cell culture. Throughout the course of this project I have perused well over 100 academic articles, and countless patents, tutorials, and textbooks, in pursuit of definitions of bioreactor mechanics and their effects on adherent and perfusion cell culture. Sorting through all of the information I had access to improved my reading comprehension of the jargon used in academic articles and the mechanical dialogue that is common in patents.

With a better understanding of what I was reading, I found that I was able to apply concepts from my classes to the complex material I was reading about. The equations I used for the fluid shear calculations were pulled directly from my fluid mechanics class, the equation for gravitational and centrifugal settling was from my downstream processing class and the discussion of growth phase change in cells after exposure to stress is built on my tissue engineering class.

To understand how the patents we researched functioned with fluid mechanics required far more mechanical engineering concepts than I had anticipated. Consequently, I had to relearn some concepts integral to mechanical engineering and teach myself how to read legal patent jargon. Additionally, while I did not get to make a full animation for this project, I did get some experience with using Blender to model the prototypes in a 3D environment and improve some of my computational modeling skills. Throughout my completion of this report, I have had the

opportunity to apply a wide range of technical and theoretical concepts I learned, or taught myself, to something tangible that I am passionate about.

In addition to getting to apply my classes to my research, I got to dive deep into the computational side of research in a way that I am unable to do in class or my own research. Class computations usually involve generalized models, and my research is largely physical tissue culturing. The computational side of this project was a daunting task, and required me to learn concepts that are glossed over in my classes. In this regard, I was able to use my contacts with my mentor to identify resources I could use to further my understanding of computational fluid dynamics. After familiarizing myself with the new content, I had to evaluate the shear equations I had learned and identify which one fit the set of assumed parameters I was working with.

Back at the beginning of the project, our evaluation of the feasibility to fabricate and settling efficiency of our proposed device designs involved calculating settling rates and critically evaluating the cost and benefit of each design for a lab to use. The cost benefit analysis was important to the industry representative we were working with because our device was a solution to a real-world problem the company R&D department had. We got to work closely with Thermo Fisher employees to get near constant feedback on what criteria is important for the design of the device and what materials are available in typical industry R&D departments. At the end of the semester, we presented our finished engineering capstone project to them and received quite a bit of critique for not focusing more on the effect of these devices on cell-culture and how they could be prepared for cell-culture use.

When writing this report, I endeavored to address their critique and cover all aspects of cell-culture in bioreactor systems I could find literature on. It is my hope that the devices presented in this report could be used in industrial and lab settings for proof-of-concept

experiments that require long culture time with media changes. As the materials are relatively cheap, with access to a 3D printer, they can be made within a day and autoclaved to be sterile for use with contamination sensitive bioreactors.

When starting a capstone, I urge students to plan ahead as far as they can and build in time for things to go wrong, have contingency plans for when resources are unavailable or a course load is more work than expected. I also recommend staying in contact with your mentor through good and bad news so they can celebrate and problem solve with you. Most of all, be flexible when the unexpected happens (like a pandemic closing down campus for half a semester) and ask for help when you need it.

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APPENDICES

Appendix A

Table 1. Decision matrix created by capstone design team and filled out by Thermo Fisher Scientific representatives. The total of each design determines how the design fits into the parameters of the project and how well it completes the overall objectives of the project.

Decision Matrix												
Design Criteria	Rating	Weight (%)	Helical Tube		Inclined Settler (commercial)		Inclined Settler (modified)		Cyclone Separator		Venturi Separator	
			Rating	Weight Score	Rating	Weight Score	Rating	Weight Score	Rating	Weight Score	Rating	Weight Score
Cost (material and manufacturing)	5.3	0.067	2	0.134	8	0.537	7.000	0.470	7.000	0.470	1.000	0.067
Process Analytical Technology (PAT)	5	0.063	5	0.317	5	0.317	5.000	0.317	5.000	0.317	4.000	0.253
Shear rates	10	0.127	3	0.380	9	1.140	8.000	1.013	7.000	0.887	0.000	0.000
Durability	6.66	0.084	8	0.675	9	0.759	9.000	0.759	8.000	0.675	2.000	0.169
Flow rates	9.66	0.122	8	0.979	8	0.979	9.000	1.101	8.000	0.979	0.000	0.000
Ease of Use	7	0.089	5	0.443	10	0.887	9.000	0.798	8.000	0.709	2.000	0.177
Time (Production)	5	0.063	5	0.317	8	0.507	8.000	0.507	5.000	0.317	5.000	0.317
Efficiency (Power reqs.)	6	0.076	8	0.608	7	0.532	7.000	0.532	6.000	0.456	7.000	0.532
Efficiency (Microcarrier seperation)	10	0.127	9	1.140	9	1.140	9.000	1.140	10.000	1.267	0.000	0.000
Scalability	8.66	0.110	6	0.658	9	0.987	9.000	0.987	10.000	1.097	2.000	0.219
Overall Footprint	5.66	0.072	6	0.430	6	0.430	6.000	0.430	9.000	0.645	4.000	0.287
Total	78.94	1.000	65	**6.082**	88	**8.215**	86.000	**8.055**	83.000	**7.819**	27.000	**2.022**

Hydrocyclone Design #1

Ratios based on diameter of 2 in (D = 2 in).

Inlet height:

$$\frac{H}{D} = 0.5 \rightarrow \frac{H}{2\ in} = 0.5 \rightarrow H = (2\ in) \cdot (0.5) \rightarrow H = 1\ in$$

Diameter of overflow:

$$\frac{D_0}{D} = 0.5 \rightarrow \frac{D_0}{2\ in} = 0.5 \rightarrow (2\ in) \cdot (0.5) = D_0 \rightarrow D_0 = 1\ in$$

Length of vortex finder:

$$\frac{S}{D} = 0.5 \rightarrow \frac{S}{2\ in} = 0.5 \rightarrow (2\ in) \cdot (0.5) = S \rightarrow S = 1\ in$$

Length of body:

$$\frac{L_b}{D} = 1.5 \rightarrow \frac{L_b}{2\ in} = 1.5 \rightarrow (2\ in) \cdot (1.5) = L_b \rightarrow L_b = 3\ in$$

Length of cone:

$$\frac{L_c}{D} = 2.5 \rightarrow \frac{L_c}{2\ in} = 2.5 \rightarrow (2\ in) \cdot (2.5) = L_c \rightarrow L_c = 5\ in$$

Diameter of underflow:

$$\frac{D_u}{D} = 0.375 \rightarrow \frac{D_u}{2\ in} = 0.375 \rightarrow (2\ in) \cdot (0.375) = D_u \rightarrow D_u = 0.75\ in$$

Hydrocyclone Design #2

Value of 2.5 mm for D_u from Ahmed (2005) dissertation.

Diameter of underflow:

$$\frac{D_u}{D} = 0.375 \rightarrow \frac{2.5\ mm}{D} = 0.375 \rightarrow \frac{2.5\ mm}{0.375} = D \rightarrow D = 6.66\ mm$$

Inlet height:

$$\frac{H}{D} = 0.5 \rightarrow \frac{H}{6.66\ mm} = 0.5 \rightarrow H = (6.66\ mm) \cdot (0.5) \rightarrow H = 3.33\ mm$$

Diameter of overflow:

$$\frac{D_0}{D} = 0.5 \rightarrow \frac{D_0}{6.66\ mm} = 0.5 \rightarrow (6.66\ mm) \cdot (0.5) = D_0 \rightarrow D_0 = 3.33\ mm$$

Length of vortex finder:

$$\frac{S}{D} = 0.5 \rightarrow \frac{S}{6.66\ mm} = 0.5 \rightarrow (6.66\ mm) \cdot (0.5) = S \rightarrow S = 3.33\ mm$$

Length of body:

$$\frac{L_b}{D} = 1.5 \rightarrow \frac{L_b}{6.66\ mm} = 1.5 \rightarrow (6.66\ mm) \cdot (1.5) = L_b \rightarrow L_b = 10\ mm$$

Length of cone:

$$\frac{L_c}{D} = 2.5 \rightarrow \frac{L_c}{6.66\ mm} = 2.5 \rightarrow (6.66\ mm) \cdot (2.5) = L_c \rightarrow L_c = 13.32\ mm$$

Inclined Settler

Diameters of inlet (to control flow rates)/outlet turned out to be negligible – controlled by pumps. Assume, $V_1 = 1\frac{L}{s},\ V_2 = \frac{2}{3}\frac{L}{s},\ V_3 = \frac{1}{3}\frac{L}{s}$; velocities dependent on diameters, velocity will be determined by pressure differential so the port size is arbitrary.

$$V_1 = V_2 + V_3 \rightarrow for\ 100 = 66.\overline{66} + 33.\overline{33} \therefore V_1 = \frac{2}{3}V_1 + \frac{1}{3}V_1$$

$$V = A \cdot u, \qquad A_{circle} = \frac{\pi}{4}d^2$$

$$\therefore V_1 = V_2 + V_3 \equiv A_1u_1 = A_2u_2 + A_3u_3 \ \rightarrow \ A_1u_1 = \frac{2}{3}A_1u_1 + \frac{1}{3}A_1u_1$$

$$\frac{\pi}{4} \cdot d_1^2 \cdot u_1 = \frac{\pi}{4} \cdot d_2^2 \cdot u_2 + \frac{\pi}{4} \cdot d_3^2 \cdot u_3 \ \rightarrow \ d_1^2 \cdot u_1 = d_2^2 \cdot u_2 + d_3^2 \cdot u_3 \equiv V_1 = V_2 + V_3$$

$$\therefore V_1 = \frac{2}{3}V_1 + \frac{1}{3}V_1 \equiv d_1^2 \cdot u_1 = d_1^2 \cdot u_1 + d_1^2 \cdot u_1 \ \rightarrow \ 1\frac{L}{s} = \frac{2}{3}\frac{L}{s} + \frac{1}{3}\frac{L}{s}$$

$$\therefore d_1^2 \cdot u_1 = 1, \qquad d_2^2 \cdot u_2 = \frac{2}{3}, \qquad d_3^2 \cdot u_3 = \frac{1}{3}$$

$$d_1^2 = d_2^2 + d_3^2 \ \rightarrow \ d_1 = \sqrt{d_2^2 + d_3^2} \ \rightarrow \ d_1 = \sqrt{\frac{2}{3} + \frac{1}{3}}$$

$$d_1 = 1, \qquad d_2 = 0.82, \qquad d_3 = 0.57$$

$$Using\ a\ inlet\ diameter\ of\ \frac{3}{4}in \ \rightarrow \ d_1 = \frac{3}{4}in, \qquad d_2 = \frac{1}{2}in, \qquad d_3 = \frac{1}{4}in$$

$$Later\ modified\colon \quad d_1 = \frac{1}{2}in, \qquad d_2 = \frac{1}{4}in$$

Lamella Separator

The dimensions were based off of having five interior plates each measuring 5 in by 5 in set at a 60° angle from the horizontal. To determine the height of the left side wall and the interior separation wall, the vertical height of the inclined plates was calculated using the equation:

$$sin(60°) = \frac{x}{5\ in} \rightarrow x = 5\ in \cdot sin(60°) \rightarrow x = 4.33\ in$$

An inch was then added to the vertical height that was calculated resulting in the dimensions of the left side wall and interior separation wall being 5 in by 5.33 in. The right-side wall is set at a 60° angle from the horizontal and must have a vertical height of 5.33 in. The length of this wall was calculated using the equation:

$$sin(60°) = \frac{5.33\ in}{x} \rightarrow x = \frac{5.33\ in}{sin(60°)} \rightarrow x = 6.15\ in$$

Therefore, the dimensions of the right-side wall were equal to 5 in by 6.15 in. The five interior plates plus the interior separation wall results in six sheets each at a thickness of 0.093 in:

$$6 \cdot 0.093\ in = 0.558\ in$$

The left and right outside walls and the six interior sheets created seven gaps that were equally spaced at the bottom at 0.625 in each:

$$7 \cdot 0.625\ in = 4.375\ in$$

The bottom length of the front and back panels needed to equal the thickness of the interior plates plus the sum of the gaps between each plate:

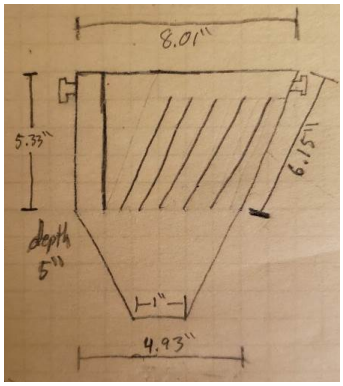
$$0.558\ in + 4.375\ in = 4.93\ in$$

The top length of the front and back panels was then calculated by using the equation:

$$tan(60°) = \frac{5.33\ in}{x} \rightarrow x = \frac{5.33\ in}{tan(60°)} \rightarrow x = 3.08\ in$$

$$3.08\ in + 4.93\ in = 8.01\ in$$

A collection chamber was then designed by creating two sets of trapezoidal pieces. The left and right-side pieces had a top length of 5 in and a base length of 1 in. The front and back pieces had a top length of 4.93 in and a base length of 1 in. Each piece was symmetrical with the sides of each piece set at a 60° angle from the horizontal.



Venturi Settler

Using ½ in inlet diameter tube, and settler tube diameter of 1 ¼ in

$$A = \frac{\pi}{4} \cdot d^2$$

$$A_1\dot{V}_1 = A_2\dot{V}_2 \rightarrow \frac{\pi}{4} \cdot d_1^2 \cdot \dot{V}_1 = \frac{\pi}{4} \cdot d_2^2 \cdot \dot{V}_2 \rightarrow \frac{\pi}{4} \cdot (1.27\ cm)^2 \cdot \dot{V}_1 = \frac{\pi}{4} \cdot (3.175\ cm)^2 \cdot \dot{V}_2$$

$$1.267\ cm^2 \cdot V_1 = 7.917\ cm^2 \cdot V_2 \rightarrow \frac{1.267\ cm^2}{7.917\ cm^2} \cdot V_1 = V_2 \rightarrow 0.160 \cdot V_1 = V_2$$

$$\therefore 16\%\ of\ flow\ in = flow\ through\ settler$$

Volumetric inlet flow rate governs convective currents in the unit which affect microcarrier settling in the unit. Decided upon 90 mL/min (11.29 cm/min flow velocity) to restrict internal flow to less than microcarrier settling velocity (12-14 cm/min).

The return rate of 0.45 mL/sec (30 mL/min) or 1/3 of inlet flow is within desired constraints for operating capacity for a 50 L reactor media exchange.

Inlet and outlet locations were decided upon as a creative decision. As the fluid flows into the settler, turbulence would be generated equally above and below the inlet. Having additional length above the inlet would facilitate a greater efficiency in settling the microcarriers and preventing them from exiting in the harvest stream. Further checking in literature shows that most designs for wastewater treatment and sedimentation vary in the dimensional placements of inlets and outlets in relation to each other[43,52,53].

Cell Shear [Honors Capstone Extension]

Assuming a steady-state condition with a dilute solution of microcarriers in a Newtonian fluid that behaves like water at 20°C and 101.3 kPa with fixed volumetric flow of 90 mL/min, viscosity, and density we can calculate the Reynold's number of the system.

The Reynold's number is a unitless number that can determine if a point in a system has laminar or turbulent fluid flow – the determination of laminar or turbulent flow is important in determining the fluid shear stress in the system. Laminar flow would see the fluid moving in a unidirectional manner which leads to less fluid shear stress from particles interacting with each other or with the materials and spaces they travel through. Turbulent flow indicates that the fluid is moving in a multidirectional manner within the space – this creates friction stresses between the particles and higher shear stress between the particles and the materials and spaces they travel through. Turbulent flow would generate eddies which would disrupt the settling process of the microcarriers in solution.

We assume the gravitational settling systems have laminar flow because that would allow for the microcarriers to settle out of solution much faster than with turbulent flow, but to evaluate if that assumption is correct we must calculate the Reynolds number of the system. This calculation is based on the flow of a microcarrier-solution as it travels through the tubing to a settling device. The microcarriers are more likely to travel along the surface of the tubing and experience shear stress than they are to experience shear stress as they settle out of solution in the settling devices. Using the 90 mL/min flow rate, we can determine the flow behavior of the system at the highest volumetric flow rate we achieved with the technology available to us during this project.

$$Re = \frac{du\rho}{\mu} \rightarrow Re = \frac{(0.00422m) * \left(\frac{90\,{}^{mL}/_{min}}{60s * 1000000\,{}^{mL}/_{m^3}} \right) * 998.2\,\frac{kg}{m^3}}{\pi * \left(0.00422\,{}^{m}/_{2}\right)^2}$$

$$0.001002\,{}^{kg}/_{ms}$$

$$Re = 450.856 \ll 2100 \ \therefore flow\ is\ laminar$$

Because the flow is laminar, we can assume there are no eddies created by the fluid flowing from the tubing into the settling devices, or even from the tubing back to the bioreactor. As the exit velocity of the microcarriers in solution is much lower than the inlet, we assume that the Reynold's number at the exit of the system is laminar as well. Thus, the shear stress of the fluid acting on the microcarriers reduces to zero throughout the system.

The wall shear stress is how the fluid interacts with the surface of a pipe (or tube) it is flowing through. If the wall shear stress is high enough, when a microcarrier bumps into it, the resultant stress could be enough to detach cells or change their growth phase. The wall shear stress can be calculated if we assume a typical laminar shear stress distribution in the tubing and maximum velocity at the centerline.

$$\tau_w = \mu \frac{\partial u}{\partial r}\Big|_{r=R}$$

$$\frac{u(r)}{u_{max}} = 1 - \left(\frac{r}{R}\right)^2 \rightarrow \frac{\partial u}{\partial r} = u_{max}(-2\,\frac{r}{R^2})$$

$$\tau_w = -2(u_{max} * \mu) \rightarrow \tau_w = -2 \left(\frac{90\,{}^{mL}/_{min}}{60s * 1000000\,{}^{mL}/_{m^3}} \right) \left(0.001002\,{}^{kg}/_{ms}\right)$$

$$\pi * \left(0.00422\,{}^{m}/_{2}\right)^2$$

$$\tau_w = -2.149 * 10^{-4}\ Pa$$

The cumulative wall shear stress for the system is $-2.149 \cdot 10^{-4}$ Pa acting opposite the flow of the fluid (hence the negative sign). Compared to the shear stress generated by impellers and baffles used to agitate bioreactors, this stress is negligible and can be discounted. In conclusion, the shear forces generated by the gravitational settling device systems calculated with the assumptions above is not enough to cause share stress on cells attached to microcarriers to result in detachment or cell growth phase change.

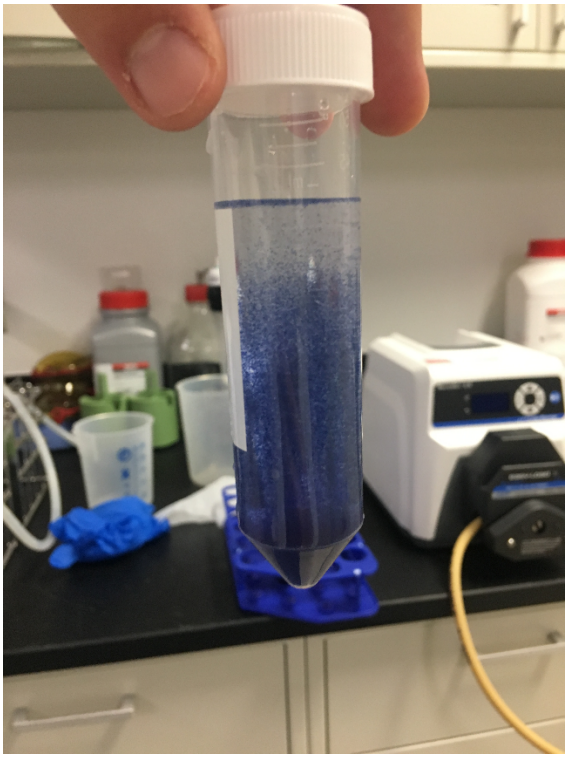
Appendix B

Protocols Developed for Project

7/10/19
Kyle Jackson

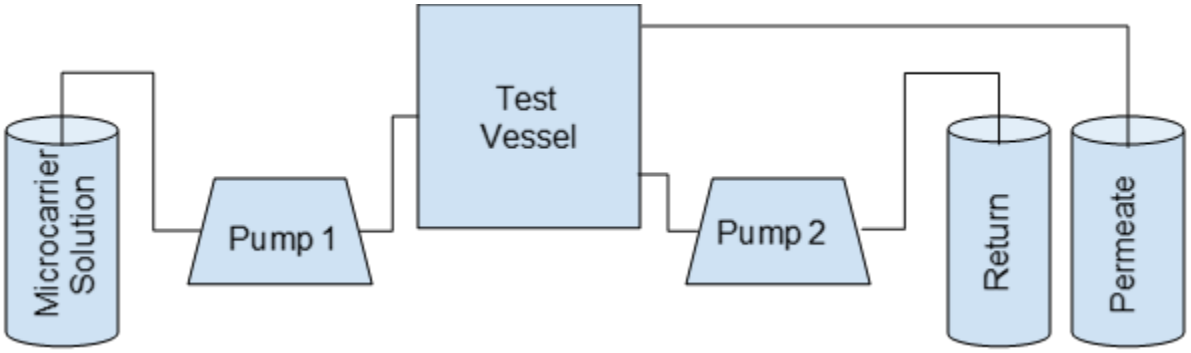
Microcarrier Dying Protocol

1. Weigh out 0.035 g of indigoidine obtained from Dr. Zhan
2. Pipette 2 mL of DMSO into a 15 mL centrifuge tube
3. Add the organic dye powder into the 15 mL centrifuge tube to dissolve the dye into the organic solvent
4. Weigh out 7 g of microcarriers and add that to the centrifuge tube. Shake the centrifuge tube VIGOUROSLY. You can also vortex the centrifuge tube. Do this until all the dye has washed around the microcarriers and there aren't any showing to be white. You can also add more DMSO to help soak up the solution
5. Let this sit for ~15mins to let the dye soak into the microcarriers
6. Then scoop out the microcarriers into the 50 mL centrifuge collector tube. Wash the collector tube with ddH_2O This will collect the microcarriers and allow the extra dye and organic solvent to wash away into the sink
7. Once the wash has turned clear, flip the collector tube over and wash the microcarriers into a new 50 mL centrifuge tube to collect the finished microcarriers
8. These microcarriers are at an undetermined concentration. Use the absorbance values or a hemocytometer to figure out the desired concentration of microcarriers



Prototype Testing Protocol

1. Measure 10 grams of microcarriers (dry weight)
2. Create 2 Liter microcarrier solution (5g/L)
3. Set pump 1 to desired inlet flow rate
4. Set pump 2 to “return percentage” of inlet flow rate
5. Fill the test vessel with DI water
6. Insert inlet tube into microcarrier solution
7. Insert return tube and permeate tube into empty containers
8. Turn on both pumps simultaneously and run 1.5 Liters through the system
9. Turn off inlet pump, leave return pump on and allow solution in vessel to empty
10. Measure and record the volume collected from the return and the permeate
11. Using microfilter cloth, filter the return and permeate solutions
12. Place filter cloth with microcarriers onto a dry paper towel
13. Weigh the filtered microcarriers (wet weight) and record the results



Example Values (Actual values entered into excel document)

Inlet Flow Rate (ml/min)	Return %	Return Flow Rate (ml/min	Return Volume	Permeate Volume	Weight of Microcarriers in Return	Weight of Microcarriers in Permeate
40 ml/min	40%	16 ml/min	16 ml	24 ml	195 mg	5 mg
40 ml/min	50%	20 ml/min	20 ml	20 ml	198 mg	2 mg
40 ml/min	60%	24 ml/min	24 ml	16 ml	199 mg	1 mg

Appendix C
Supplemental Information

Equation 1. Calculation of daily rate of media exchange based on permeate flow rate.

$$Daily\ rate\ of\ media\ exchange\ (L) = Permeate\ flow\ rate \frac{mL}{min} \cdot \frac{68400\ min}{day} \cdot \frac{L}{1000\ mL}$$

Table 1. Table with inlet and permeate (microcarrier return stream) flow rates for the three selected designs. Daily rate of media exchange was calculated using Equation 1, and is only a prediction of media exchange if the devices are left on for 24 hours.

Device	Inlet flow rate (mL/min)	Permeate flow rate (mL/min)	Daily rate of media exchange (L)
Hydrocyclone Separator	288	N/A	N/A
Lamella Separator	96	96	138.24
Inclined Settler	90	30	43.2

Table 2. Raw data for the hydrocyclone separator. The inlet flowrate was kept at a constant 90 mL/min for all tests for a comparable data set. The hydrocyclone had an overall percent recovery below the 99% project goal regardless of the permeate volume used. Data partially missing from return and permeate volume calculations.

Inlet Flow Rate (mL/min)	Return % of Inlet	Return Flow Rate (mL/min)	Return Volume (mL)	Permeate Volume (mL)	Weight of Microcarriers in Return (g)	Weight of Microcarriers in Permeate (g)	Recovery %
90	90%	81	1400	230	2.02	0.57	77.99%
90	94%	85	880	90	1.16	0.38	75.32%
90	90%	81	1346	270	5.33	2.22	70.60%
90	94%	85	N/A	N/A	6.34	1.69	78.95%
90	85%	77	N/A	N/A	25.81	2.35	91.65%
90	80%	72	N/A	N/A	21.02	6.14	77.39%
90	75%	68	N/A	N/A	20.15	7.7	72.35%
90	85%	77	N/A	N/A	22.95	5.34	81.12%

Table 3. Raw data for the lamella separator. The lamella separator was tested most efficiently at ~76 mL/min inlet flow rate. This flow rate gave a greater than 99% recovery of microcarriers from solution. The prototype gave recovery below a 90% at anything greater than 100 mL/min for the inlet flow rate. The test with pluronic and NaCl resulted in an increased recovery with a previously tested inlet flow rate.

Inlet Flow Rate (mL/min)	Return % of Inlet	Return Flow Rate (mL/min)	Return Volume (mL)	Permeate Volume (mL)	Weight of Microcarriers in Return (g)	Weight of Microcarriers in Permeate (g)	Recovery %
74.0	25%	18.50	375	1125	39.00	0.420	98.93%
76.0	33%	25.30	500	1000	33.17	0.071	99.79%
76.0	40%	30.40	600	900	53.69	0.110	99.80%
75.6	60%	45.36	900	600	35.72	0.014	99.96%
92.4	40%	36.96	600	900	49.96	0.599	98.82%
126.0	33%	42.00	500	1000	28.05	13.220	67.97%
104.2	33%	34.73	500	1000	33.58	2.721	92.50%
99.1	33%	33.04	500	1000	37.55	2.474	93.82%
92.4	33%	30.80	500	1000	31.24	0.576	98.19%
100.8	40%	40.32	600	900	44.23	3.354	92.95%
Tests with 1 g/L pluronic and 7.15 g/L NaCl							
92.4	33%	30.80	500	1000	25.89	0.035	99.86%

Table 4. Raw data for inclined separator. The percent recovery was above 99% at inlet flow rates around 60 mL/min. Convective forces caused the microcarriers to flow into the return stream more often at flowrates above 90 mL/min.

Inlet Flow Rate (mL/min)	Return % of Inlet	Return Flow Rate (mL/min)	Return Volume (mL)	Permeate Volume (mL)	Weight of Microcarriers in Return – including water weight (g)	Weight of Microcarriers in Permeate (g)	Recovery %
90	33%	30	500	1000	29.2	1.12	96.31%
90	33%	30	500	1000	29.08	1.28	95.78%
120	33%	40	500	1000	34.4	1.37	96.17%
120	33%	40	500	1000	34.27	1.46	95.91%
150	33%	50	500	1000	30.8	2.94	91.29%
150	33%	50	500	1000	37.1	3.99	90.29%
60	33%	20	500	1000	20.96	0.015	99.93%
60	33%	20	500	1000	44.25	0.023	99.95%
60	33%	20	500	1000	43.27	0.019	99.96%
90	25%	22.5	375	1175	22.24	0.69	96.99%
120	25%	30	375	1175	18.26	1.17	93.98%
150	25%	37.5	375	1175	29.4	3.9	88.29%
90	40%	36	600	900	33.56	0.38	98.88%
120	40%	48	600	900	26.8	1.02	96.33%
150	40%	60	600	900	32.9	1.6	95.36%
Tests with 1 g/L pluronic and 7.15 g/L NaCl							
60	33%	20	333	666	19.2	0.0001	100.00%
150	33%	50	333	666	22.76	0.54	97.68%

Figure 1. The set up for testing design prototypes is shown. This includes two separate inlet and outlet peristaltic pumps (left and right) sandwiching the initial lamella prototype.

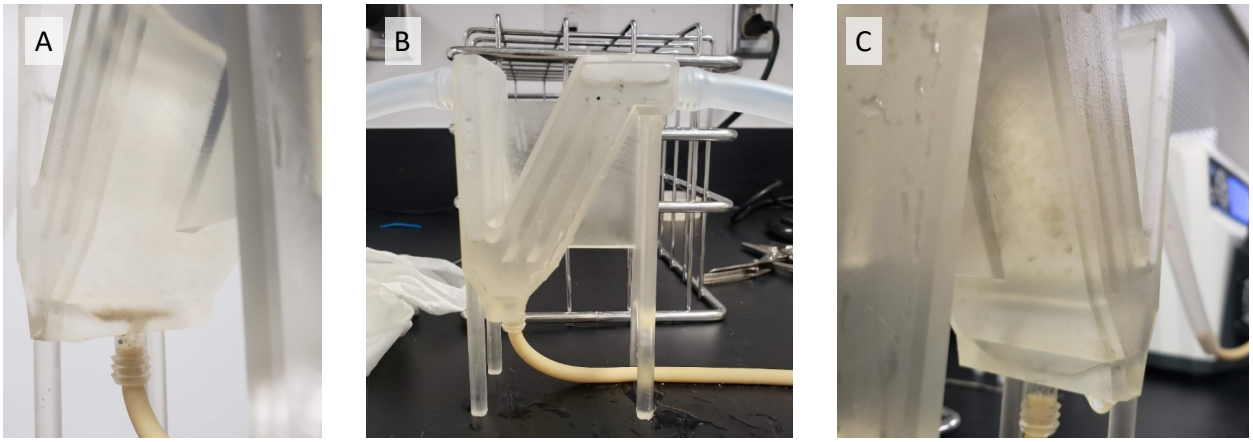


Figure 2. Microcarriers settling out as a white precipitate from the liquid in the 3D printed lamella prototype (A). Picture of lamella separator with undyed microcarriers seen in transparent incline (A). Entire 3D printed lamella prototype connected with inlet (upper tubes on B) and outlet (lower port and tube C) streams with a concentrated microcarrier stream out of the bottom (C). Sideways profile of the 3D printed Lamella inclined separator (B). Microcarriers seen adhered to the side of the prototype.

Figure 3. Dyed microcarriers separated from permeate and return streams caught on mesh from hydrocyclone tests. The microcarriers were weighted on the mesh to calculate the efficiency of separation.

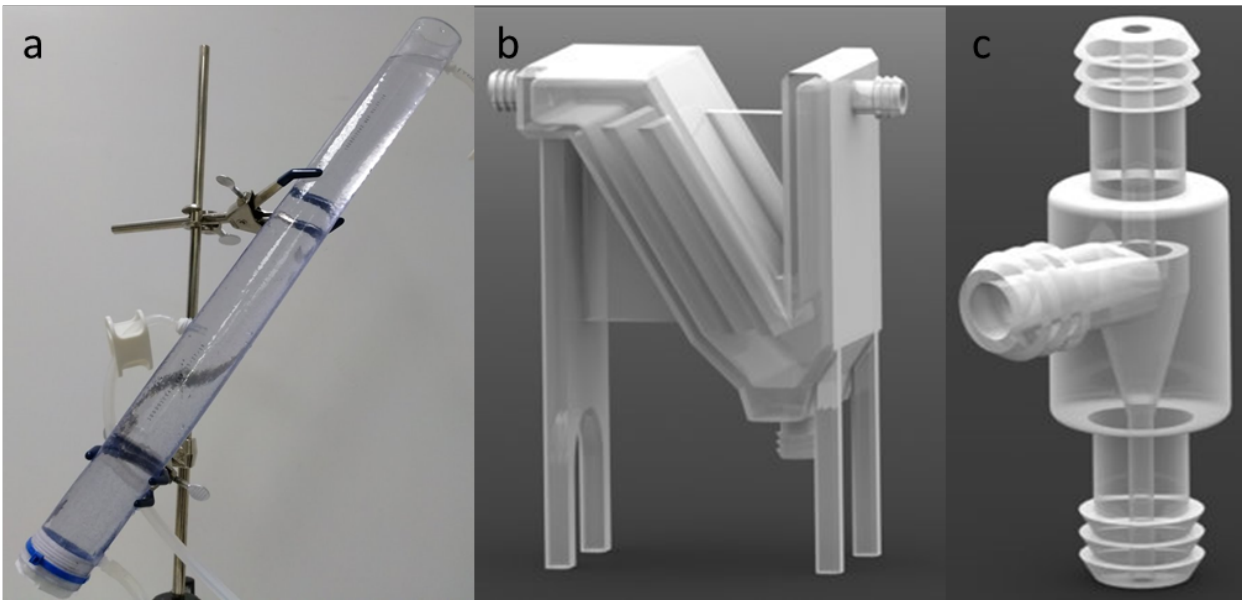


Figure 4. Prototype designs from left to right: (a) inclined settler, (b) lamella separator modeled in SolidWorks and (c) hydrocyclone separator modeled in SolidWorks.

PROFESSIONAL AUTHOR BIO

Tessa Burrows pursued a B.S. in Biological Engineering with minors in Mathematics and Chemistry on a Presidential Legacy Scholarship awarded on academic merit. She was an active Undergraduate Research Fellow, Honors student, and Society of Women Engineers member throughout her undergraduate. During her career at Utah State University, she received the Engineering Undergraduate Research Program and the Undergraduate Research Creative Opportunity scholarships and funding to pursue research with retinal degeneration and spider silk in the Tissue Engineering lab. She has presented her research at multiple conferences and was awarded first place in the poster presentation category at the Intermountain Biological Engineering Conference in 2019. She will be pursuing a PhD in biomedical engineering with the University of Minnesota Twin Cities with a focus in tissue engineering and regenerative medicine starting Fall 2020. Tessa hopes to improve *in vitro* laboratory systems to more accurately represent *in vivo* conditions and comprehensive tissue reactions to reduce the time needed for FDA approval of novel therapeutics in industry and academia.