Bioreactor Cassette for Autologous Induced Pluripotent Stem Cells

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Table of Contents

Abstract

Autologous induced pluripotent stem (iPS) cells show great potential for use as patient-specific medical therapeutics. In order for this therapy to be effective, it is necessary to have a bioreactor cassette system capable of providing conditions to grow multiple iPS cell samples from individual patients without exchanging media between samples. To address this issue, we created and analyzed several different cassette designs with computational fluid dynamics (CFD) to determine the best geometry to achieve consistent flow. After reviewing our CFD results, we determined that our best design for flow involved a fan into a rectangle with guides, but due to rapid prototyping limitations, this design was modified into two new designs: the straw design and the balanced runner design. The balanced runner and straw designs, along with the original rectangle design, were dye tested to analyze their fluid distribution. We determined that the straw design has the most potential to provide reliable, uniform flow throughout the cassette. Future work will optimize this design further, and integrate a bubble trap, cell metabolism monitoring, and ergonomic clamping into the cassette-bioreactor interface.

Background

iPS Cells

Stem cells, through which any of the body's 200 cell types are derived, are a source of great therapeutic potential. Their power comes from their pluripotency, or the ability to be maintained indefinitely in culture until signaled to differentiate into a specific, specialized cell type (NIH, 2010).

Most public controversy surrounds the use of human embryonic stem cells (hESCs), or lines derived from the inner cell mass of a 3–5-day old blastocyst. Fortunately, induced pluripotent stem (iPS) cells, or adult somatic cells reprogrammed back into a pluripotent state, have been developed. These cells avoid this ethical obstacle and by their nature make stem-cell therapy a more individualized practice (MedicalNet, 2010).

Stem cell-based therapeutics, or treatments in which induced differentiation of stem cells is used to repair damaged or destroyed cells or tissues, is showing great progress in its potential for use in a clinical setting (NIH, 2010). Until recently, stem cell growth has been used primarily to model cell growth and function, or to perform drug screening. As the potential for implantation and use in a patient approaches, however, researchers are experiencing a need to grow stem cells under individually controlled conditions in specialized culture chambers called bioreactor cassettes.

Bioreactor cassettes are small, specialized culture dishes used to maintain undifferentiated colonies of stem cells. They are usually attached in a network to a bioreactor (Appendix A), which incubates and provides them with a continual, though variable, supply of media appropriate for growth (Hei, 2010). Later, they can be subjected to specific chemical or mechanical signals that direct their differentiation.

As patient-specific therapy becomes a clinical reality, it will be necessary to have a bioreactor cassette capable of providing conditions to grow multiple iPS cell samples from individual patients without exchanging media between samples.

Problem Statement

Autologous iPS cells show great potential for use as patient-specific medical therapeutics. The most common approach to iPS cell growth is to take a skin biopsy and grow the cells in conditions that induce pluripotency; they can later be controllably differentiated into the desired cell type. However, for this therapy to be cost-effective, it is necessary to have a bioreactor cassette capable of providing conditions to grow multiple iPS cell samples from individual patients without exchanging media between samples. We will design a cassette and perfusion interface to address these emerging demands.

Current Solutions and Competition

Due to the novelty of the field of stem cell therapeutics, stem cells have not been cultured on a large scale in an undifferentiated form. However, since cells have been used in the biotechnology industry for a long time now, there are many methods, bioreactors, and cassettes that offer competition.

Currently, the simplest cell culture solution is to grow the cells statically. In static cell culture, the cells adhere to the bottom of a flask and are constantly bathed in culture media. Media replacement is performed in bulk. Static cell culture flasks can be purchased from Corning as shown in Figure 1. While static cell culture is a common way to grow small quantities of cells, especially for research purposes, there are many limitations associated that render it useless for large-scale
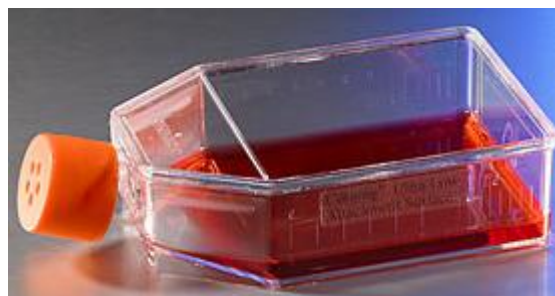

Figure 1: Static cell culture flasks sold by Corning (Product #3814) are frequently used to grow cells. The cells are attached to the bottom and are bathed in liquid media as shown.

Image Courtesy of Corning
http://www.corning.com

iPS cell culture. First of all, for the cells to remain undifferentiated, they require constant supply of autocrine factors, which would be removed when the entire media is exchanged (Zandstra and Nagy 2001). In addition, the buildup of waste products leading up to the media exchange could cause differentiation or inefficient self-renewal (Zandstra and Nagy 2001). Finally, to be useful in large-scale therapeutic applications, it would be better to be able to grow the cells with less human intervention.

The biotechnology industry has found solutions to the mass production problem associated with static culture. In many mass-production, biotechnological applications for cell culture, the cells are grown in a batch mode stirred flask bioreactor (Zhang *et al.* 2010). In this system, the cells are grown in suspension until they reach a high density. As cells start to die from lack of nutrients and too much waste, they are harvested for the desired product. In order for most cells to grow in suspension, they must be adapted to lose anchorage-dependence. This adaptation alters the cells in a way that makes them undesirable to be implanted back into the body as the loss of anchorage-dependent growth

is associated with malignant cancers. An additional problem associated with this traditional method is that the harsh conditions of nutrient-starvation, growth in suspension, and waste accumulation would likely cause stem cell differentiation (Zhang *et al.* 2010).

One solution to some problems associated with the batch-mode stirred flask bioreactors is to use a perfusion bioreactor. In a perfusion bioreactor, fresh liquid media is constantly run through the cassette, allowing efficient waste removal and nutrient addition. Many variations of perfusion bioreactors have been employed to culture cells, and many of them involve growing the cells in suspension with a method for separation of cells from media at the output. One patent on such a perfusion bioreactor solves the separation problem by enclosing the cells in a semipermeable bag (impermeable to cells) (Singh 2003). However, because the cells must still be adapted to growth in suspension first, this method would not be ideal for growing iPS cells for therapeutic purposes. Another perfusion bioreactor that has been patented solves the problem of having to grow the cells in suspension by placing microcarrier beads in the medium, which allow the cells to attach, similarly to a normal tissue culture substrate, but to still get the nutrients, oxygen, etc. that they require for healthy growth (Schwarz and Wolf 1992). While this system alleviates the problem of anchorage-dependence, these harsh conditions are likely to cause differentiation of stem cells and the cells may not be exposed to factors secreted by other iPS cells, also promoting differentiation.

Perhaps the most significant competing solution for us, however, is a cell culture cassette that is designed to interface with a perfusion bioreactor. This CLINIcell cassette can be used to grow adherent cells (Figure 2). It provides a better solution than those listed

Figure 2: The CLINIcell cassette provides the most competition to our design proposal in that it allows perfusion culture with adherent cells. However, the geometry of the cassette is not optimized for flow distribution, the plates are too thin, allowing oxygen permeability, and the cassette size is too small for our purposes

Image Courtesy of INNOMEDITECH
http://www.innomt.com/

previously, but it fails to provide a perfect solution for our problem. First of all, the rectangular shape with input and output valves on top does not optimize the flow characteristics, resulting in dead space and possibly causing stem cell differentiation in some areas. The thin plates of this cassette provide another concern due to their oxygen permeability: the iPS cells we intend to design our cassette for require low oxygen content in the media and the slow perfusion rate allows oxygen to diffuse into the media if the cassette is permeable. Additionally, the cassette does not provide enough area to grow cells. Finally, if this cassette were to be used in a parallel operation, it would need to have a way to monitor the cell growth and change the perfusion rate of individual cassettes in order to compensate for different growth rates.

Ethical Considerations

The knowledge that cells grown in these bioreactor cassettes may be permanently inserted into the human body is of primary ethical concern. Cell growth and activity, especially as induced from a pluripotent state, is extremely sensitive to external stimuli. During material selection, process testing, and flow analysis, our team will pay special attention to the presence of material extractables, conditions under which extractables are more likely to form and interact with the cells, and motion dynamics which might encourage prolonged cellular exposure to extractables or other factors that might cause an undesirable outcome. In this way, we can ensure that cells eventually differentiated and ultimately used for research or clinical therapeutics are of the highest, most natural quality possible, and are therefore as safe as possible for the patient.

Along with safety, it will also be important that the bioreactor cassette affords security to the patient receiving the therapy or medical staff administering the therapy. Any commercial product should be disposable, to limit chances of patient-to-patient cross-contamination or sample alteration due to poor sterilization.

We will also consider the individual nature and patient specificity inherent to iPS cells. Cells from different patients do not necessarily grow at identical rates, so the design must allow for observation, as well as lag time in the feeding schedule of each sample, so that individual cultures are given the best chance to grow successfully. This avoids multiple visits or biopsies of the patient, and limits unnecessary suffering and expense.

Finally, it will be important to develop a cost-effective, safe device so that promising clinical therapeutic research and implementation can move forward efficiently and productively.

Ergonomic Considerations

The bioreactor system is built to be largely self-sufficient. Therefore, after adding the cells, interaction with the cassette will be primarily to monitor iPS cell growth. Accordingly, the bioreactor cassette will have a transparent top so that microscopic viewing can be done while maintaining sterility in the cassette. Along those lines, valve connections from the cassette to media input and output lines should be easily and securely engaged and disengaged, and a frame must be established in the incubator so that individual cassettes can be removed and replaced without disturbing nearby cassettes. Each cassette must be marked clearly with patient identification so that cultures at various stages can be attended to appropriately. In order to monitor metabolism of the cells easily, the pH monitor must be mounted so that problems can be detected visually, and regulation of flow input must be easily accomplished soon after.

Design Criteria and Considerations

Our client would like a cost-effective cassette system that allows several different samples to be connected to one perfusion bioreactor without exchange of media or cross-contamination. The cassette will be transparent and have an area of approximately 300 cm^2. It will facilitate confluent growth and adherence, encourage appropriate fluid flow coverage, and monitor the metabolism of growing iPS cells through pH. The perfusion interface will also allow variable control of flow rate and volume of nutrient media supplied to each cassette, while maintaining physiological conditions within them. Both components will be sterilizable with multiple autoclave cycles, composed of polymers not known to affect stem cell fate, and disposable. The cassette and interface will be designed such that
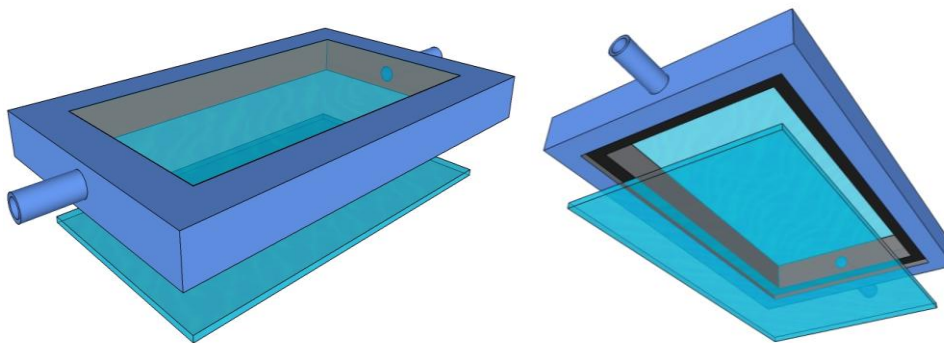
Figure 3 – Top (left) and bottom (right) view of cassette design concept. The cassette consists of a frame and cell growth plate. Both the top of the frame and cell growth plate are transparent.

sterility can be maintained if the cassettes need to be removed from the bioreactor for microscopic viewing (See Appendix B). The cassette will consist of two main parts – a frame and cell growth plate (Figure 3). The frame will have luer lock connections for input and output of media and have geometry that creates consistent flow across the entire cassette. The cell growth plate will lock into the bottom of the frame to allow a surface for the iPS cells to grow and proliferate on. Both the cell plate and top of the frame must be transparent so the researcher can visually monitor the growth of the cells.

Materials

There are a few requirements materials must meet in order for them to work for this device. First, there must be no extractables that could cause cell death, differentiation, or any other negative effects. Using USP Class VI certified or Food Grade materials will help avoid this problem. The second material prerequisite is that the cell growth plate and topside of the frame must be transparent. This allows easy visual monitoring of the cells. The final requirement is that the material must be sterilizable – specifically by autoclaving since it is the cheapest and easiest method in most labs. We also will keep in mind that gamma sterilization would be a more ideal method in a mass production situation.

We have decided to use tissue culture polystyrene for the cell growth plate. The appropriate shape will be cut out of a Corning 500 cm² Cell Culture Dish (Cat. # 431110) to fit into the cassette's frame and then resterilized. This material will be ideal since it is USP Class VI certified and already used to grow iPS cells successfully.

Two different materials are being considered for the cassette's frame: Nylon and PC-ISO (polycarbonate). These materials were selected because they are USP Class VI certified (Stratasys Inc, 2009; 3D Systems Inc, 2007) and are compatible with rapid prototyping. Additionally, both materials have been proven to withstand multiple cycles in an autoclave through testing. We have also tested their compatibility with H9 embryonic stem cells: six semi-circular disks of each material were created and placed in a 6-well plate with the cells for three days. Growth was scored and it was determined that the materials did not negatively impact the cells. A potential problem with both of these materials is transparency – nylon is opaque and PC-ISO is only translucent. Another transparent material, such as glass or Lexan, might be inserted into the center of the top frame, solving this potential setback.

To provide a tight seal between the frame and cell plate, we have decided to use a silicone gasket. Silicone is commonly used in medical fields and many formulas are USP Class VI certified (Columbia Engineered Rubber Inc., 2010). The silicone selected has been provided by our client because of its previous use with stem cells.

Flow

Flow is a very important aspect of this design – irregular or forceful flow patterns such as eddy currents could cause enough mechanical force on the iPS cells to cause differentiation. Additionally, dead spots in the flow would starve and kill cells in those areas. Geometry of the cassette will primarily govern the fluid dynamics within it. The height of the cassette must promote minimal use of media without creating high shear forces on the cells, which could lead to undesired differentiation (Zhang *et al.* 2010). To find the best flow pattern, different geometric designs were evaluated using a computational fluid dynamics (CFD) computer simulation program, CFX through Ansys 14.0.

All of the following designs give a maximum of 23x15x2 mm cellular growth volume. Each input and output valve is designed to taper from a 3 mm diameter luer locking source to a 2 mm diameter circular opening in the cassette. See Appendix C for dimensions of each design.

Simple Rectangle

The simple rectangle design (Figure 4) is the easiest and most obvious solution to the problem. The rectangle is already employed in some static culture systems as it maximizes the amount of cell growth due to its larger surface area. For our prototype, the rectangle would make production the easiest as the cutting of the cell plate shape requires skilled use of a hot-wire device. Negatively, more media perfuses through

Figure 4: The simple rectangle is easy to prototype and maximizes surface area but does not yield the best fluid flow.

the larger volume of space, so this design likely will not be as efficient at reducing media use. The biggest concern with this design is that there may be dead space in the corners.

Diamond

The diamond design (Figure 5) was conceived in attempt to guide the flow of fluid toward the edges from the input valve to the output valve. Cutting this design from a pre-purchased polystyrene cell plate would likely be as easy as the simple rectangle. The design also has a similar cell growth surface area to the simple rectangle, though slightly smaller.
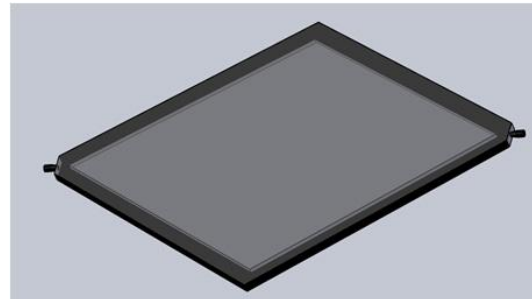

Figure 5: The diamond is also easy to prototype but also does not yield the best fluid flow.

Ellipse

An elliptical shape (Figure 6) was designed as it was thought to achieve the same function as the diamond without the harsh corners which might collect eddy currents. The gentle slope of the curve might guide the flow to the peripheral frame was well as the interior of the plate. The
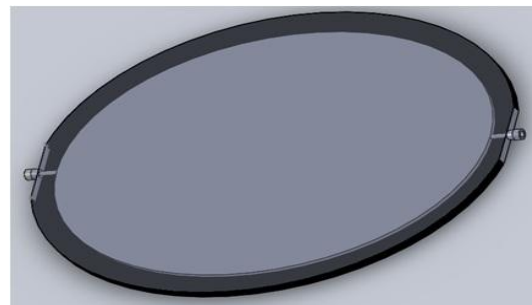

Figure 6: The ellipse geometry is difficult to prototype but may yield good fluid flow while minimizing media waste.

smoothing of the edges also maximizes the efficiency of media changes per day. Unfortunately, this design would be very hard to cut accurately from pre-purchased polystyrene without the use of a water jet.

Fan into Rectangle with Flow Straighteners

The fan into rectangle model with flow straighteners (Figure 7) was created from a combination of the diamond model with current static culture systems. The sloped part of the "fan" guides the flow to the periphery, until it reaches the straighteners, where flow is pointed in the correct direction to perfuse to the outlet. These flow straighteners, however, add considerable difficulty to the production of the prototype as they cannot be attached to the pre-purchased polystyrene cell growth plate, but must be fabricated as a component of the frame itself. Additionally, the surface area in which the cells can grow would be reduced at the expense of a small total cassette size, as it is intended for cell growth to occur in the region following the flow straighteners only.

Figure 7: The fan into rectangle with flow straighteners is difficult to prototype and wastes some media.

Fan into Rectangle with Guides

The fan into rectangle with guides (Figure 8) directs fluid to the periphery while simultaneously straightening flow. This approach saves space compared to the fan into rectangle with flow straighteners, but is more intricate, and would require a more precise fabrication technique. Accidental occlusion of one lane might be more likely, as a result of the very narrow entry of the fluid from the input valve into the fan guides. Cutting the cell growth plate for our prototyping purposes would remain as easy as the rectangle because cell growth would only occur after the flow
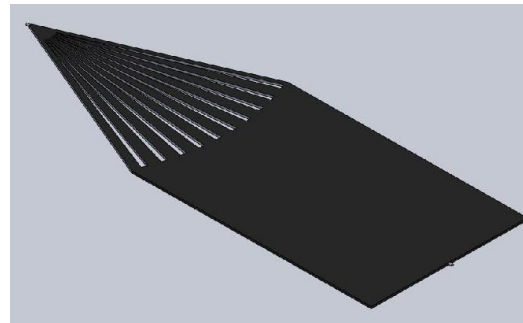

Figure 8: The fan into rectangle with flow straighteners is more difficult to prototype and wastes some media but may yield the best fluid flow.

guides. The use of media is not as conserved as other designs due to the increased volume of space taken up by the flow guides.

Computational Fluid Dynamics Analysis

After deciding on designs to analyze, we used computational fluid dynamics (CFD) with CFX (using Ansys 14.0 software). These calculations are inherently dependent on the boundary conditions: we chose a no-slip wall boundary condition for all surfaces except the inlet and outlet, set the inlet velocity to be the velocity needed to go through 3 volume changes per day (which was less than 1 mm/s for all of the cassette geometries we analyzed), and we set the outlet pressure to be 0 Pa. We used these boundary conditions for both velocity contour and streamline analyses.

The velocity contours can be seen in Appendix D. Overall, we were looking for the velocity to be as consistent as possible over the cell growth area. The rectangle geometry velocity contours reveal many areas of concern, including areas of abrupt velocity change. The diamond geometry seems to fare better than the square, with fairly consistent velocity in the center, with minor problem areas on the edges. Contrary to our *a priori* expectations, the ellipse velocity contour map exposes many areas with variable velocity. Assessing our more complicated geometries—the fan with straighteners and the guided geometry—we find that the velocity contours look much more promising for the guided geometry. It is important here to point out that the cells would only be grown on the cell plate, which would be placed downstream of the straighteners. Therefore, the velocity contour prior to the cell plate is not a variable that we are particularly concerned with optimizing. Overall, the velocity contour analyses point to the guided geometry as having the best flow characteristics.

We also used CFD to look at the streamlines through each geometry (Appendix D). We analyzed each cassette using 100 streamlines with the same boundary conditions as previously. Perhaps the most convincing reason that we think that the guided model has the best flow characteristics is because it indeed works as intended, guiding the flow outward. The streamlines show that the flow is being directed outward toward the edges in this model, whereas, in the others, the streamlines remained centered near the middle of the cassettes.

Input Valve

The cassette will provide a stable and secure attachment to the bioreactor via luer locks. Since the rate of autocrine factors and waste products released from the cells changes throughout the proliferation process, the rate of media must be variable. This change will be done either manually with a valve system (limiting the amount of the media going to some cassettes, while maintaining higher perfusion for others) or automated with a computerized system. Additionally, maintaining sterility is a concern for disconnecting and reconnecting the cassette for visual inspection, and this must be addressed in our future work.

Monitoring Systems

In order to know when the cells are ready for use, the design requires a monitoring system. One way this can be done is through pH. As the proliferation processes continue, the output media decreases in pH due to increases in lactic acid production from the cells (Zandstra and Nagy 2001). Therefore, if the pH of the output media can be measured, the state of the cells can be monitored. Fiber Optic pH Sensors such as those found with Polestar (Polestar Technologies, Inc., 2009) and Ocean Optics (Ocean Optics, 2010) could be connected to the media output of the cassette to do such monitoring . This data would then be sent to a computer which would alert the researcher that the cell growth in a certain cassette has reached an optimal level.

Design Evaluation

All of the flow designs were evaluated on a scale of one to ten and weighted on a variety of design criteria (Table 1). The flow analysis was determined to be the most important design characteristic and given the most weight because an irregular flow could cause negative cellular effects such as cell death or differentiation. Feasibility of prototype, maximizing cell production, and efficient media use were the next highest considerations because all of these aspects affect cost-efficiency of the design; we want to be able to

maximize cell growth while minimizing price. Mass production was determined to be less important, as this characteristic does not cause many problems with modern production technology. Based on the results, the fan into rectangle with flow guides was most favorable and therefore is the design we chose to pursue.

Table 1 - Design matrix that displays our evaluation on a scale of one to ten and weighted on a variety of design criteria for all design concepts.

	Weight	▭	◇	○	⬡	⇒
Fluid Analysis	0.60	4	7	6	8	9.5
Feasibility for prototype	0.15	10	9	4	9	9
Mass–Production	0.05	9.5	9	9	9	9
Maximize Cell Production	0.10	10	9	9	10	10
Efficient Media Use	0.10	8	8	8	7	7
	Score	6.18	7.70	6.35	8.30	9.20

Prototyped Designs

Considerations for Prototyping

Due to our CFD analysis and prototyping limitations, we altered our chosen flow guides design to better fit these accommodations. Based on the CFD analysis, guiding the flow from the inlet to the cell growth plate of the cassette positively impacted the consistency of the flow. Therefore, we made sure our final design incorporated this aspect. However, the fan with guides design needed to be altered due to rapid prototyping limitations and the size of the gasket used to seal the plate onto the cassette. Combining these considerations, we chose to ultimately pursue a new design we denote the "straw" design. In addition to the straw design, a new design called the balanced runner design was also prototyped.

Another important aspect to incorporate was a method of attaching a cell plate to the cassette. While this plate will eventually be made of tissue culture polystyrene, we used a Lexan plate for testing purposes. To seal the plate to the cassette, a silicone o-ring gasket was used. A groove was designed around the cell growth area in order to set the gasket. Finally, screw holes were tapped in on the sides of the cassette in order to clamp the plate onto the cassette.

While the silicone gasket solved the problem of sealing the plate onto the cassette, incorporation of the gasket groove posed another problem that we needed to address in our designs: the gasket groove occluded the flow. We addressed this issue by directing the flow around the gasket in two different ways, as will be discussed with the individual designs (*vide infra*). Changes were also made to attempt to optimize the outlet area of the cassette.

Rectangular Design

We chose to pursue the rectangular design (Appendix E) in addition to the more complicated geometries in order to compare the flow characteristics. In addition to the changes described above to accommodate prototyping, we made changes to the inlet and outlet from a simple rectangle. Due to the silicone o-ring gasket used to seal the plate onto the cassette, we directed the flow from the inlet with sharp 90˚ turns to get around the gasket (Figure 10). At the outlet, this design involved a trench to be used as a collecting trough before the fluid enters the outlet (Figure 11).
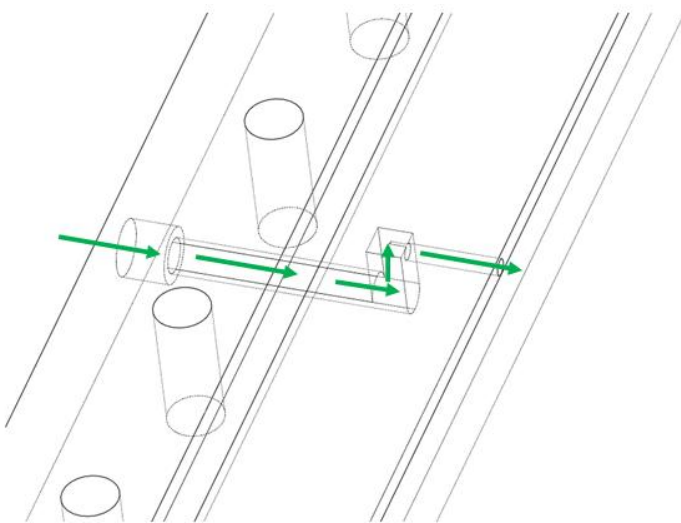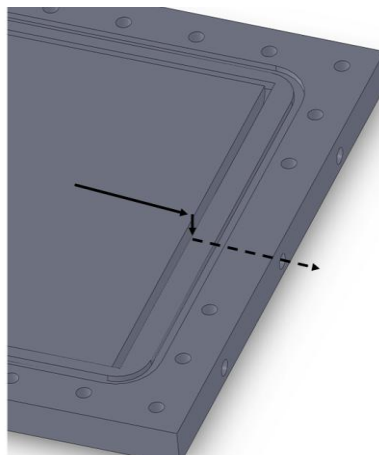
Figure 10: Close-up of the inlet of the rectangular cassette, showing the sharp angles used to go around the gasket. Green arrows indicate the path of flow from the inlet to the cell growth area.

Figure 11: Close-up of the outlet design for the rectangular cassette. This design involves dropping the flow off into a collecting trough, as shown by the black arrows.

Straw Design

We expected that the straw design (Appendix E) would behave similarly to the CFD data we found for the flow guides design due to physical barriers guiding the flow from the inlet to the outlet. Based on the complexity of this design, however, we have not been able to complete CFD analysis of this design due to program limitations that we have not yet been able to overcome. In the initial design, we intended to vary the cross-sectional area of the hole of the cylinders based on having each cylinder accept the same percentage of the fluid flowing in. As the flow went from the middle of the cassette toward the edges, the cross-sectional area of the cylinders' holes increased due to the

Figure 12. Numbered cylinders in the straw design.

Table 2: The diameter of the hole at the top of each cylinder in the straws design, with each cylinder numbered from the center outward to the edges as shown in Figure 12.

Cone	Diameter (mm)
1	1.27
2	1.41224
3	1.54432
4	1.6637
5	1.77546
6	1.8796
7	1.9812
8	2.07518
9	2.16662
10	2.25298
11	2.3368
12	2.41808
13	2.49682
14	2.57302
15	2.64668
16	2.7178
17	2.78892
18	2.85496
19	2.92354
20	2.98704

decrease in total fluid flow leftover (since the previous cylinders allowed some of the fluid to leave the main tube). However, in the end, prototyping limitations forced us to remain within 1.27 mm to 3.11 mm diameter range due to the sensitivity of the equipment, the size of each layer of material, and the necessity of cleaning out the resin from the straws after prototyping. Within the constraints of this range, we varied the size of the cylinders from the middle to the outside based on the cross-sectional area of the cylinders. The diameter of the cylinders and their placement can be found in Figure 12 and Table 2.

The inlet and outlet of the straws design differs from that of the rectangular design. For the inlet, rather than 90° turns, we were able to angle the straws upward, going underneath the gasket. At the outlet, we used a design similar to the collecting trough in the rectangular cassette, with a slight modification. This trough was arched in the hopes of minimizing turbulent flow and to aid in bubble removal.

Balanced Runner Design

Another design that was tested was the balanced runner design (Appendix E). This design is conceptually quite different from the straw design. While the straw design relies on the differing cross-sectional areas of the inlets, the balanced runner is based on the principle of equally splitting the flow multiple times. Theoretically, this equal splitting results in the same percentage of the flow going to each leg, balancing the "balanced" runner. An additional design consideration here was the velocity of the flow in each leg. In order to increase the velocity further within the runner, the design has smaller cross-sectional area in each successive leg. Each new branch of the runner decreases in cross-sectional area by a factor of two (Appendix E). Ultimately, this difference in cross-sectional area should result in a velocity twice as fast in each successive leg since velocity of the flow should be inversely proportional to the cross-sectional area of the path. Thus, this forces the fluid out of the runner and into the cell growth area. The inlet and outlet characteristics of the balanced runner design are not changed from the rectangular design.

Testing

Set Up

Dye testing was used to verify appropriate distribution of fluid within the cassette. The cassette was placed in a darkened room on an illuminated surface, and secured underneath a PlantCam time-lapse camera. Neutral density filters were placed to ensure that a consistent light intensity reached the camera as it was recording fluid patterns within the cassette, and care was taken to keep the system undisturbed for the duration of the dye study.

A peristaltic pump with an EW-07519-20 pump head was attached to the cassette to provide fluid input analogous to that supplied by a bioreactor, and calibrated to ensure appropriate flow. Microbore tubing was fitted onto the pump and the luer lock connection of the cassette. The pump regulated flow input at a constant exchange rate of 3L/L/day. This flow rate varied

Figure 13. Example of dye experiment setup. Cassette was placed on illuminated surface and secured underneath a time lapse camera. A pulsatile pump supplied a flow rate of 3 fluid exchanges per day.

depending on the volume of the cassette used (*Table 3)*. Components, such as the output draw, were placed at cassette height to avoid siphoning effects (*Figure 13*.)

After the cassette was filled entirely with 0.9% saline solution, clear fluid supply was exchanged for colored supply with a maximum absorption of 628.5 nm to optimize contrast. The subsequent flow distributions within the cassette were recorded once per minute by the time-lapse camera for approximately 24 hours, and analyzed in iMovie. Saline solution was used in place of cell media to save cost when visualizing flow and due to the similar viscosity to cell growth media (Derek Hei).

Table 3 – The flow rate supplied to the cassette varied slightly depending on the volume of the cassette to give an exchange rate of 3 L/L/day.

Cassette	Volume of Cassette (mL)	Flow Rate Supplied (µL/min)	Pump Speed (rpm)
Rectangle	143.87	299.8	17.4
Straw	121.03	252.2	15.5
Balanced Runner	81.66	170.1	11

Results and Observations

The rectangle, balanced runner and straw designs were all tested. Due to prototyping limitations, the balanced runner guides were not sealed on their topside, leaving a significant void of communal fluid above them. Fluid from the guides that leaked into this void affected flow. Therefore, two perfusion experiments were conducted with this design: the first performed with this void present, the second performed after this void was sealed with a silicone gasket. One dye test run was performed with the rectangle and straw designs.

Dye Test Results

Rectangle
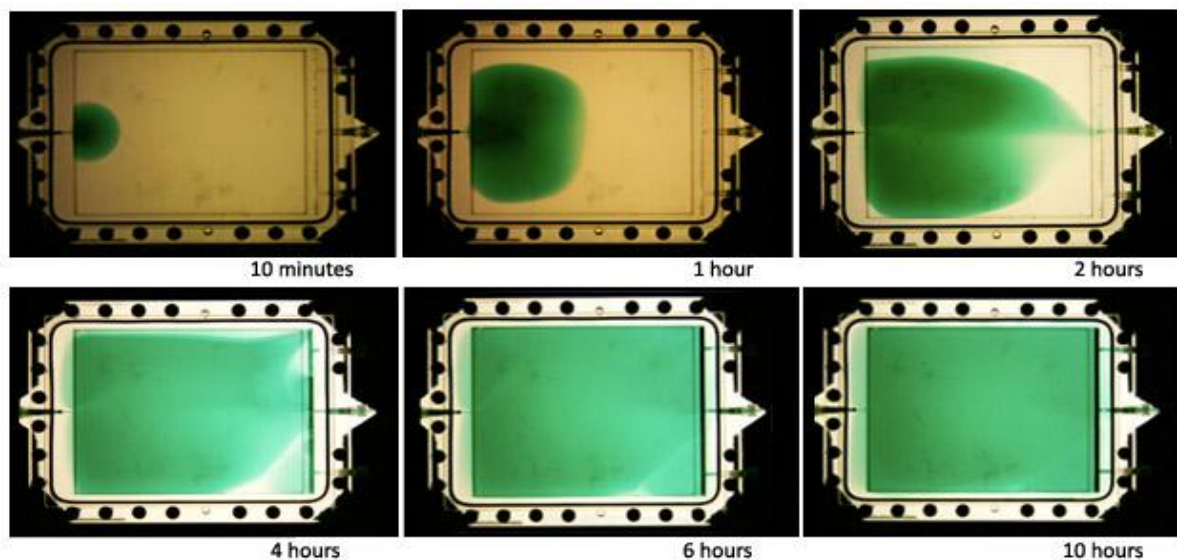
Figure 14. Representative sampling of flow distribution in the rectangular design. A circular front morphs into a bullet which pushes through body of cassette until contact is made with the outlet. Several hours pass before dye contacts the lateral walls. Only central outlet allows fluid to exit, others are plugged.

The rectangular cassette was supplied with dye at a velocity of 299.8 μm/minute. A circular front of fluid mushrooms slowly from the inlet, continuing as a bullet-like progression of dye throughout the body of the cassette (Figure 14). Dye was distributed to the lateral walls slowly. Initial contact of the main body of fluid with the first (lower) lateral wall was made 2 hours after perfusion began - after the main bullet-like front of dye had

reached the middle of the cassette body. Contact with the second (upper) lateral wall was made approximately 5 hours after perfusion began - 3 hours after the main front had reached the outlet. Note the capillary draw that takes place near the outlet, as well as the diffusion that takes place to dye the corners near the outlet of the cassette. It takes 7 hours to fully perfuse the rectangular cassette.

Balanced Runner Design, Void Present

The balanced runner cassette was supplied with dye at a velocity of 170.1µm/minute. A definite central front of fluid travels through the body of the cassette, and capillary draw pulls fluid toward the edge of the cassette after initial contact is made (Figure 15). Note the diffusion of dye above the balanced runner guiding region, as well as
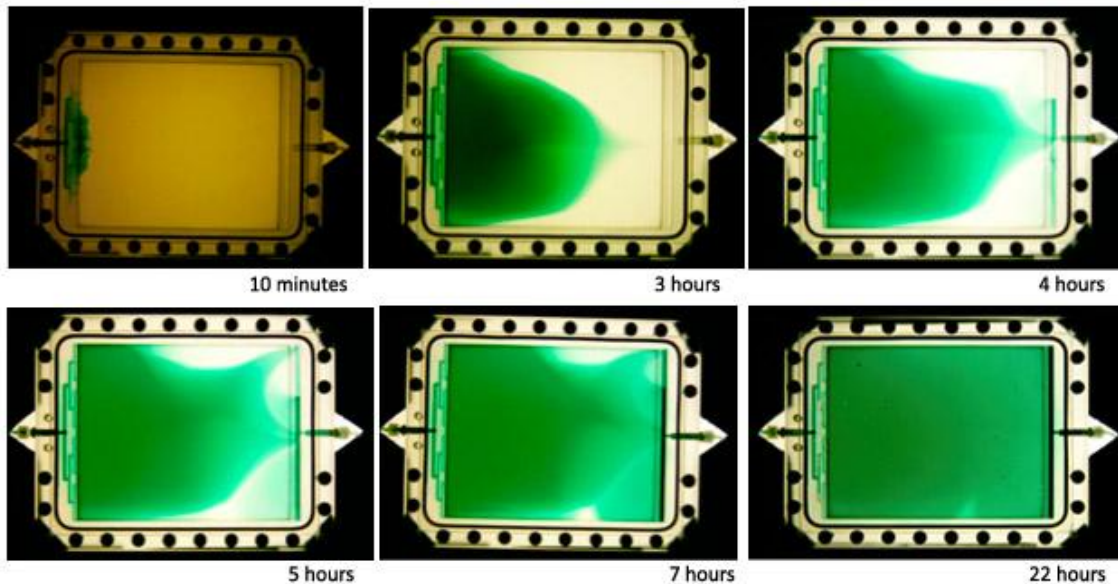
Figure 15. Representative sampling of flow distribution in the balanced runner design, with incompletely sealed balanced runner guides. A centralized front pushes through body of cassette until contact is made with edge of cassette or trough, which guides dye distribution toward the contacted edge, or laterally with diffusion back into the body.

lateral distribution of dye and subsequent diffusion back into the body of the cassette after front reaches the trough near the outlet of the cassette. It took 22 hours to observe complete perfusion of dyed fluid in the cassette.

Balanced Runner Design: Void Sealed with Gasket

The guiding section of the balanced runner cassette was sealed with a silicone gasket, and the cassette was supplied with dye at a velocity of 170.1μm/minute. A centralized front is observed, but it is not as distinct as in the balanced runner with the void present (Figure 16). Note lateral distribution of dye and subsequent diffusion back into the body of the cassette after front reaches the trough near the outlet of the cassette. It took 16 hours to observe complete perfusion of dyed fluid in the cassette.
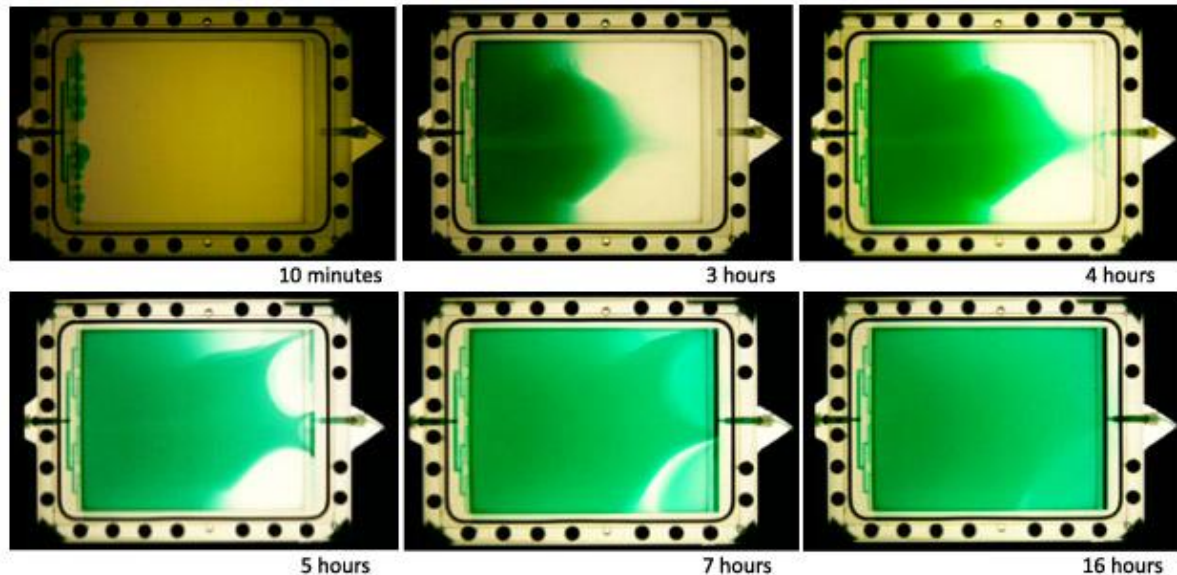
Figure 16. Representative sampling of flow distribution in the balanced runner design, with sealed balanced runner guides. A relatively uniform front pushes through body of cassette, until contact is made with edge of cassette or trough, which guides dye distribution toward the contacted edge, or laterally with diffusion back into the body, respectively.

Straw Design

The straw design was supplied with dye at a velocity of 252.2 μm/minute. Deformation (bowing upward) of the center of the lower plate skewed collected results, though useful information about the initial behavior of the straws and their reaction to bubbles can be described (Figure 17). It seems that two initial fronts emerge from each group of straws, and dye slowly diffuses back into the central region of the cassette. It is difficult to determine if this slow diffusion toward the center is a result of the gap in straw placement at the center of the array, or the bowed nature of the center of the cell growth area. Most likely it is a combination of both.
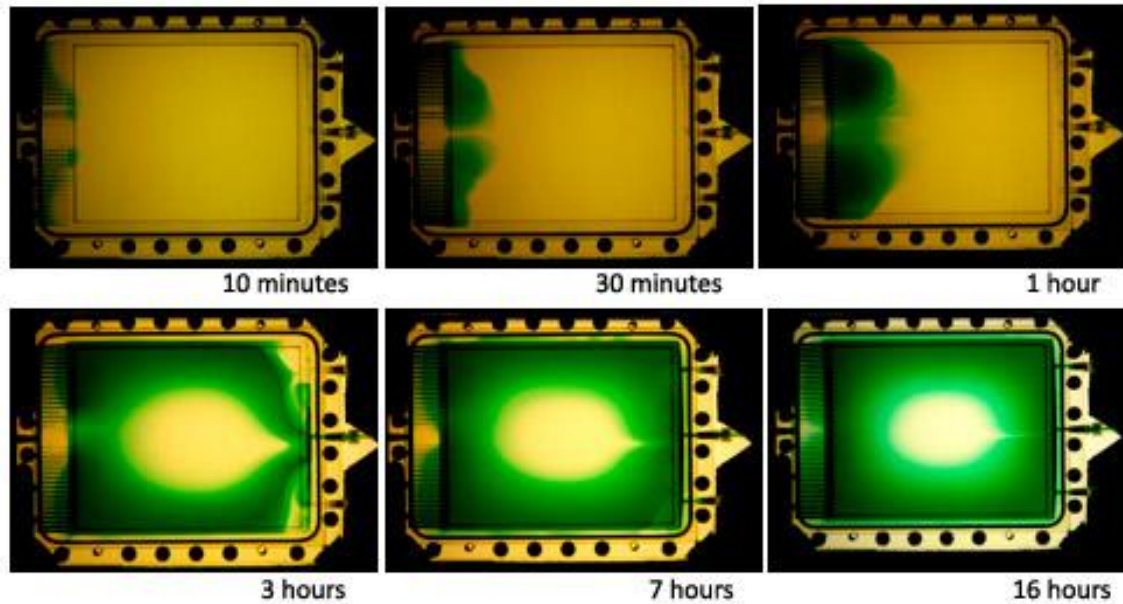
Figure 17. Representative sampling of flow distribution in the straw design. Deformation in the center of the cell growth plate disrupts flow pattern, leaving a dead zone in the center of the cassette.

Observations

Bubble Formation

Bubble formation occurred within all cassettes tested. It is suspected that bubbles are both introduced in the media, and form within the cassette itself. In the balanced runner design, bubbles lodged within the flow-guiding region had the potential to significantly affect flow distribution in the cassette over time (Figure 18). In region A (Figure 19), complete perfusion was observed after 8 hours, but complete perfusion of

Figure 18. Bubbles lodge in the network of the balanced runner design, can affect flow, and are difficult to remove. Note lack of fluid delivery to section blocked by bubble.

region B, which was supplied by a network with a bubble lodged, was still not evenly perfused after the 24 hour recording period.
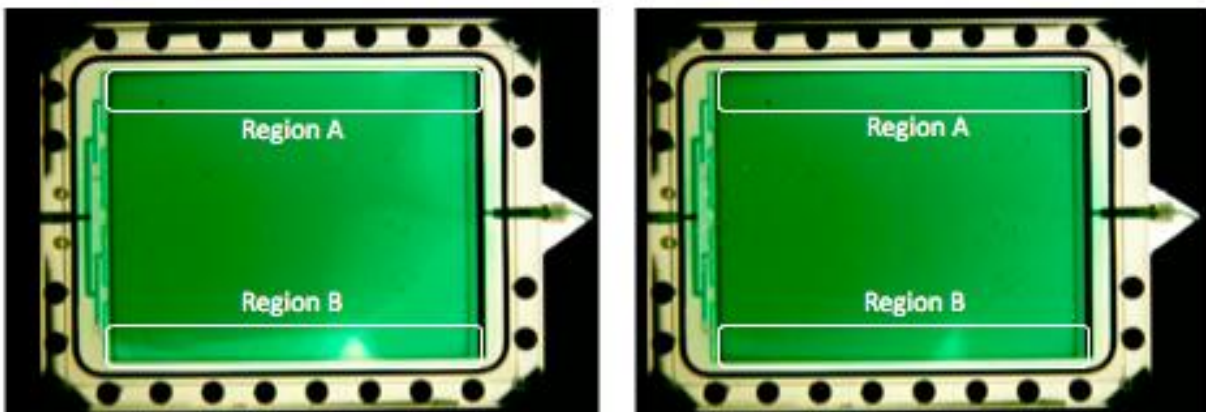
Figure 19. Balanced runner without gasket network that supplies region B is blocked by a bubble. Incomplete perfusion of the area is observed and maintained for 15 hours after comparable region (Region A) has completely perfused. Left: perfusion after 8 hours. Right: perfusion after 23 hours.

Bubbles formed within the straw design as well, but were not as detrimental to cassette perfusion as bubbles within the balanced runner. Although flow is stopped by bubbles in the innermost straws (1.27mm diameter), it is retained despite bubble formation within the outer straws (Figure 20).
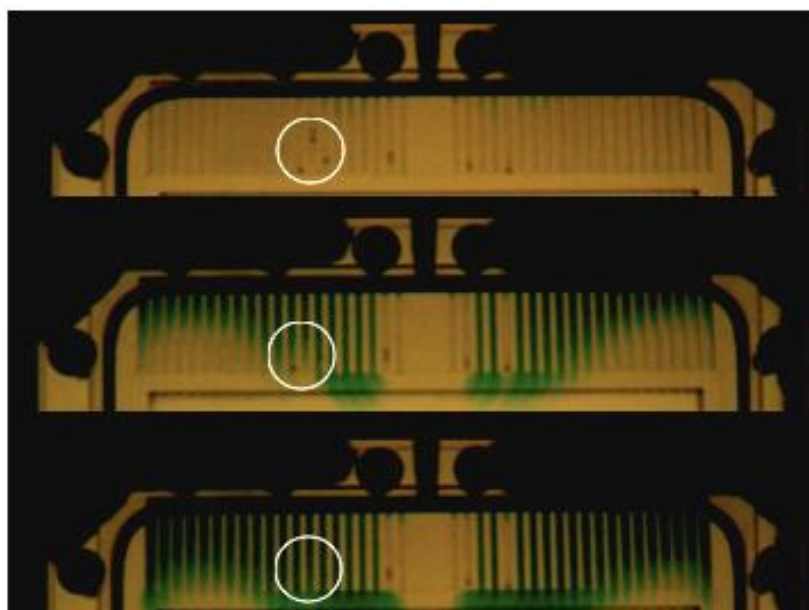
Figure 20. Bubbles existing in the outer straws of the straw design do not completely occlude flow. Bubbles in the inner straws do significantly occlude flow. Top: perfusion after 1 minute. Middle: perfusion after 10 minutes. Bottom: perfusion after 18 minutes.

Lateral Flow

Dye testing experiments revealed interesting fluid distribution due to the trough designed near the outlet of the cassette. Upon reaching the outlet region, dye was observed to follow the path of least resistance, and rapidly diffused laterally, and back into the body of the cassette (Figure 21). This effect varied between cassette designs, and was especially prominent in the balanced runner with void design.
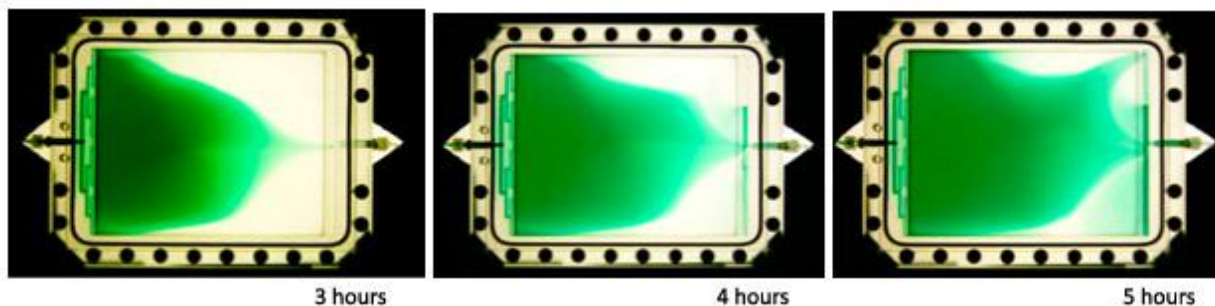

Figure 21. Left: Observation of lateral flow in balanced runner with void. Initial contact with the outlet of the cassette by the central front (3 hours). Middle: Lateral diffusion of fluid was observed near the outlet of the cassette, over trough (4 hours). Right: Significant diffusion from the trough region back into the cassette is observable (5 hours).

Cassette Durability

After prolonged exposure of the cassette to fluid, peeling within the cassette body occurred. Attempts were made to even the surface, but a slightly rough surface may have been imparted by this delamination, and may have affected observed fluid flow patterns – though we suspect that the impact was minimal.

As a response to this peeling in the balanced runner design, lacquer was applied to the newly fabricated rectangular and straw designs. In both of these prototypes, inward bowing of the lower (cell growth) area of the plate was observed and obscured results. The team speculates that this misshapen lower plate could be due to one or more of the following:

1. inaccurate fabrication
2. unevenly applied lacquer, with an increased thickness in the center
3. chemical qualities of the lacquer reacting adversely with the heat of the light source

4. undue pressure applied to the cassette wall when it is initially filled with fluid. Further testing will elucidate the cause of this deformation.

It seems also that tightening of the screws near the inlet has the potential to bow the cassette inlet and slightly skew observed flow distribution. Therefore, great care was taken to maintain uniform compression between the body of the cassette and covering plate between flow experiments.

Conclusions

The rectangular design provides a baseline for flow distribution in a single flow inlet, single flow outlet cassette design. Although complete perfusion was achieved relatively rapidly (presumably a result of the increased cassette volume and thus flow rate that the absence of a guide system affords), the design poorly distributed flow to the lateral edges of the cassette. The flow pattern of this design was unlikely to be significantly disturbed by the presence of bubbles.

The balanced runner design is an innovative approach to dividing flow before it enters the cell growth area. Dye testing showed that it improved supply to the cassette periphery, but there is still a clear leading front in the center of the cassette. This pattern of flow leaves dead spaces in the lower corners of the cassette, which are eventually perfused by back-diffusion from dye that accumulates in the outlet trough of the cassette. Unfortunately, this design proves to be significantly disrupted by bubbles in the runners, especially in primary branches. Though we plan to introduce a bubble trap, bubbles could still potentially form beyond the bubble trap, disabling uniform flow. This risk is unacceptable in a final design.

The straw design shows promise in providing reliable, uniform flow within the cassette. Unfortunately, a complete analysis of flow uniformity was impossible because of the bowing. Because initial dye distribution suggests a flow trend similar to that of the balanced runner, but without the risk of significant disruption by bubbles, we will continue to pursue the straw design as a contender for the final solution.

Cost Analysis

During the investigation of bioreactor cassettes, expenses accrued from prototype fabrication, testing materials, and testing setup according to the following (Table 4):

Table 4: Cost analysis of Bioreactor Cassette investigation of solutions.

Unit	Unit Cost	# Units	Total Cost ($)
Prototypes			
Rectangular	862.5	1	862.5
Balanced Runner	881.25	1	881.25
Straw	881.25	1	881.25
Prototyping Subtotal			2625
Test Materials			
Original Polyamide	450	1	450
Test Polyamide	37.5	10	375
Original Polycarbonate-ISO	281.25	1	281.25
Test Polycarbonate-ISO	24.38	10	243.75
Test Materials Subtotal			1350
Testing Setup			
PlantCam	88.95	1	88.95
Density Filters	7.5	2	14.99
Adaptor Ring	9	2	17.99
Tubing	1.60/ft	3	4.8
3-way Luer Valves	23.75	2	47.5
Testing Subtotal			174.23
Total			**4149.23**

Fabrication of the prototypes was accomplished on the SLA, SLS, and FDM machines at the Milwaukee School of Engineering. A total of 53.5 hours were spent by operators there to create the rectangular, balanced runner, and straw prototypes, as well as the test materials.

Future Work

Though determining the optimal design for even flow distribution throughout the bioreactor cassette was the primary challenge in creating a useful device, we are excited to continue to develop the cassette until it is commercial viable. A complete model will need

to include a bubble trap, metabolism monitoring system, and clamp closure, as well as undergo further cell growth testing and be compatible with grid-pattern installment into a bioreactor.

Immediate future work will involve further dye testing. First, the straw design will be slightly modified by increasing the diameter of the two innermost straws on each side of the inlet to 1.54 mm, to match the diameter of the third straw, which was the first to demonstrate reliable, un-occluded flow. This modified straw design will then be tested without the central distortion, and flow pattern will be evaluated. We will also consider further theoretical analysis for determining dimensions.

We will also perform testing to determine the effects of multiple outlets on flow distribution in the straw and rectangular designs. These results will determine the final outlet configuration of the cassette.

A bubble trap will be a necessary addition to the cassette design because the media supply to the bioreactor cassette is oxygenated, and the team anticipates that bubble formation between media supply and cassette delivery will be unavoidable in a realistic system. Since bubbles have the potential to significantly alter flow patterns for large periods of time when embedded in the flow guide network, bubbles must be cleared before reaching the flow guides. We have therefore considered several alternatives to solve this problem: degassing vents, gas-permeable membranes, and cassette additions before the flow guides, which would shunt and hold bubbles for venting at a later time. In fact, we have designed a preliminary model of an external bubble trap that could be connected to the cassettes through a luer lock connection (Figure 22). The concept of this design is that the media would flow through it (prior to entering the cassette)
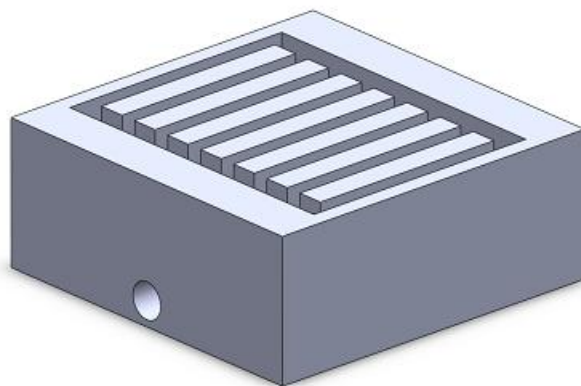

Figure 22 – Preliminary model of an external bubble trap. See Appendix F for dimensions.

and the bubbles would float to the top and be essentially trapped. Thus, the media would be allowed to flow through removing at least some of the bubbles that form prior to entrance into the cassette. Considerations with added size and sterilizability will primarily

dictate which technique for bubble removal is chosen. Sterilizability could potentially be a problem for materials selection.

After reliable flow patterns have been established and bubble interference has been eliminated as much as possible, the team can move into cell growth tests in a bioreactor setting. Cassettes will be seeded and cells allowed to adhere to a media coating on the polystyrene cell plate in the polycarbonate frame of the bioreactor cassette. The cassette can then be perfused at the appropriate rate, and cell growth patterns can be monitored using immunostaining and microscopic investigation of differentiation state. Cell viability will also be analyzed to determine dead areas within the cassette.

Meanwhile, the team will be able to begin programming and integration of a fiberoptic pH probe to monitor metabolism of the cells, and feed back to monitor flow rate to the cassette appropriately. This system would have to be tailored to each cassette individually, so commercial use of the bioreactor system could be nearly completely "hands off" after seeding of the cells takes place, therefore minimizing perturbation and possible damage to the growing cells. This step will require a modification to the inlet tubes to allow easy manipulation of the amount of media perfusing each cassette.

Finally, framing of the bioreactor cassette model must be slightly adjusted to be ergonomically friendly for the user. Instead of screws, a clamp system will be incorporated to make covering of the system faster and more consistent. Framing design will also need to be compatible with a stacking and identification system within the bioreactor incubator, so that individualized patient samples can be stored and used appropriately in a clinical setting.

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        Engineering.* 2001. 3: 275-305.

Appendix A: Bioreactor Schematic
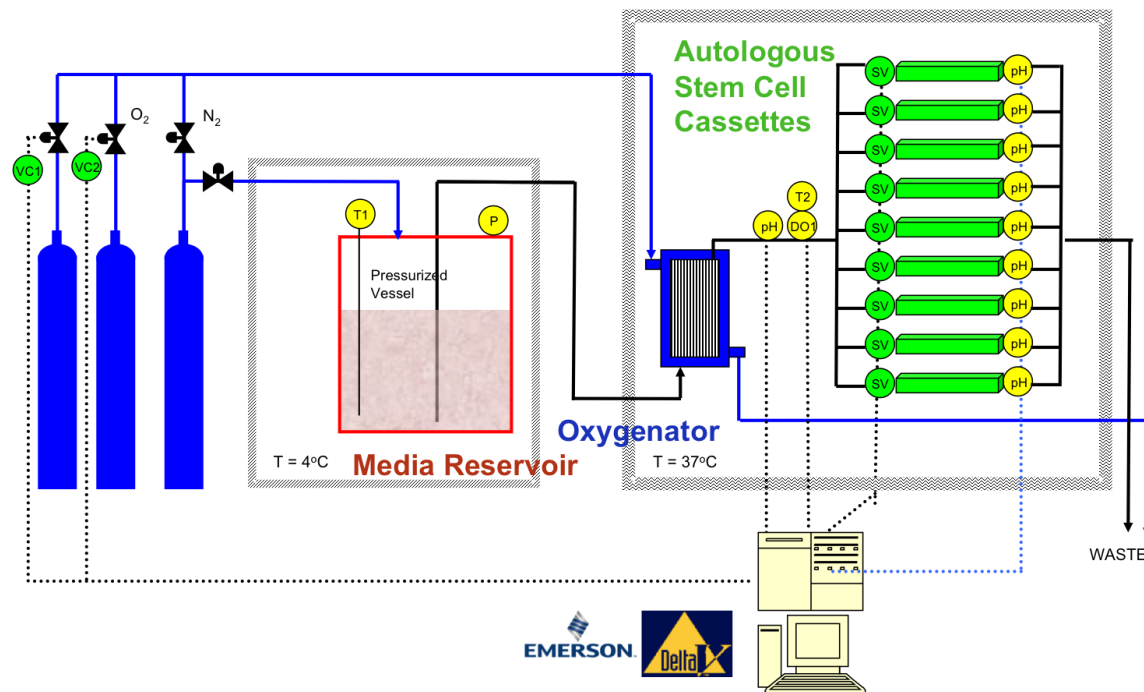
Schematic for the perfusion bioreactor used in this project. Media is perfused through cassettes in parallel so that a single bioreactor can be used without exchange of media.

Appendix B: Product Design Specifications
Project Design Specifications—Bioreactor Cassette
March 10, 2010

Team: Ali Johnson, Kim Kamer, Elise Larson, Laura Zeitler
Client: Derek Hei, PhD – Technical Director, Waisman Clinical Biomanufacturing Facility
Advisor: Willis Tompkins, PhD

Function:

The bioreactor cassette will provide appropriate conditions to culture multiple samples (from different patients) of iPS cells without exchanging media between samples. The cassette will be transparent and have an area of approximately 500 cm^2. It will facilitate confluent growth and adherence by encouraging appropriate fluid flow coverage. The perfusion interface will allow variable control of flow rate and volume of nutrient media supplied to each cassette, while maintaining physiological conditions within them. Both components will be sterilizable with gamma radiation or autoclaving, and composed of polymers not known to affect stem cell fate, and disposable. The cassette and interface will be designed such that sterility can be maintained if iPS cells need to be removed from the bioreactor for microscopic viewing.

Client Requirements:
- Steam and gamma sterilizable
- Connects to bioreactor interface and allows variable media flow
- Gas-impermeable cell growth plates
- Optically transparent
- Monitor pH
- No extractables in contact with media

Design Requirements:
1) Physical and Operational Characteristics
    a) *Performance requirements* – Must provide an appropriate cell growth environment with proficient perfusion of media.
    b) *Safety* – Must not contain any chemicals or substances that will negatively influence the cell, cell growth or initiate differentiation.
    c) *Accuracy and Reliability* – Must provide appropriate culture conditions that do not initiate differentiation
    d) *Life in Service* – Prototype: sterilizable, withstands repeated use (at least 10) and fluid submersion.
       Final product: One-time use, up to 1 month
    e) *Shelf Life* – Able to withstand a basic medical storage environment
    f) *Operating Environment* – Must work properly at 37° C and in constant exposure to a liquid media.

g) *Ergonomics* – Should not interfere negatively with the user's ability to monitor the cells.
h) *Size* – Cell growth area of 300 cm^2, depth of 2 mm
i) *Weight* – Under 1 kg/cassette
j) *Materials* – sterilizable, transparent, allow cell growth, not influence differentiation
k) *Aesthetics* – Transparent cell plate
2) Production Characteristics
    a) *Quantity* – One, but should be designed with the intent of mass production in the future.
    b) *Target Product Cost* – $400
3) Miscellaneous
    a) *Standards and Specifications* – Uses USP Class VI Materials, adheres to Good Manufacturing Practice Guidelines and Good Tissue Practices
    b) *Customer* – Medical Research Community
    c) *Patient-related concerns* – Must not negatively influence the cells

Competition – There are currently different culture systems but none that allow for several different samples with no exchange in media.

Appendix C: Dimensions for Flow Designs

Note: All dimesnsions are in mm
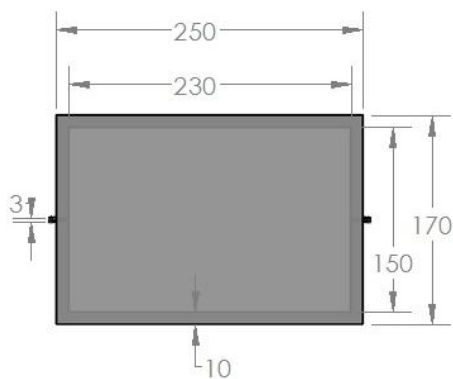
Simple Rectangle
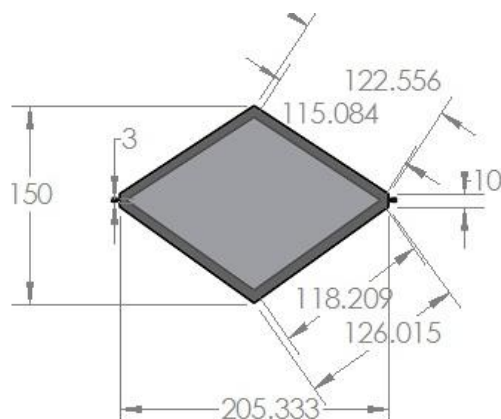
Diamond
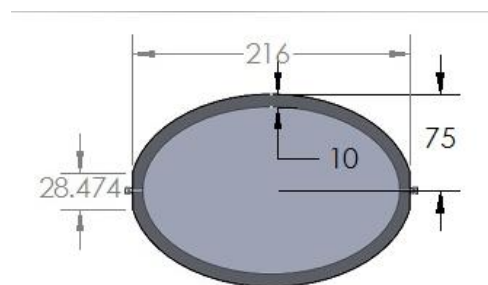
Ellipse
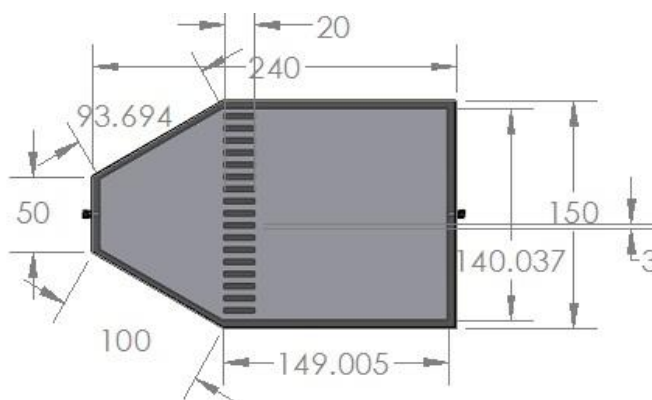
Fan into Rectangle with Flow Straighteners
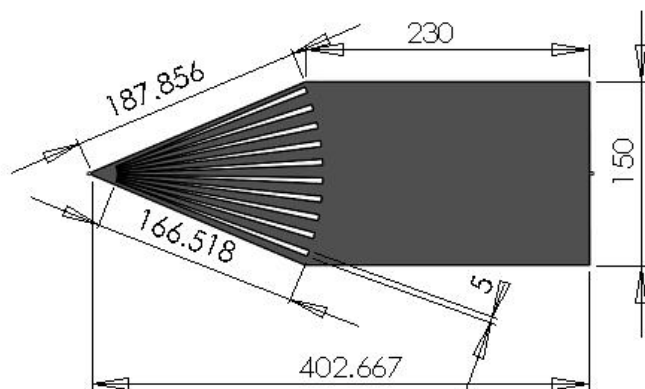
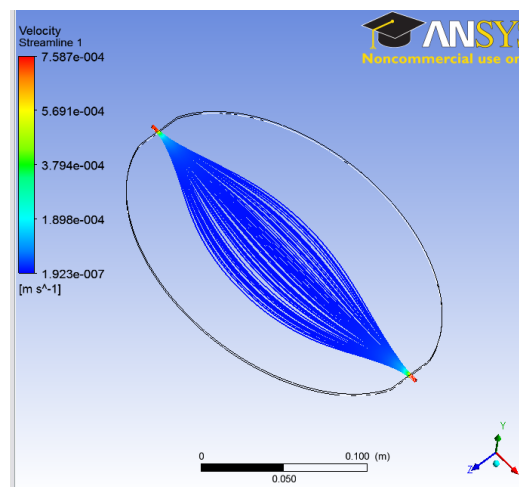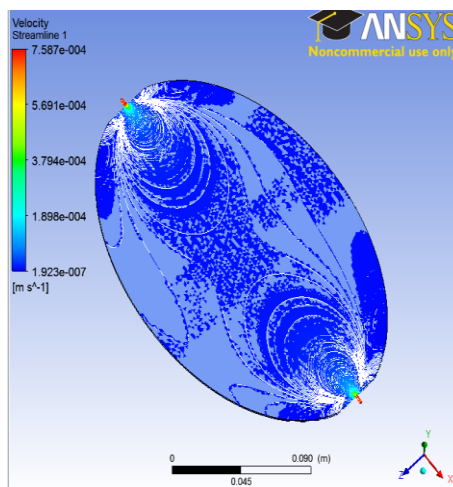Fan into Rectangle with Guides
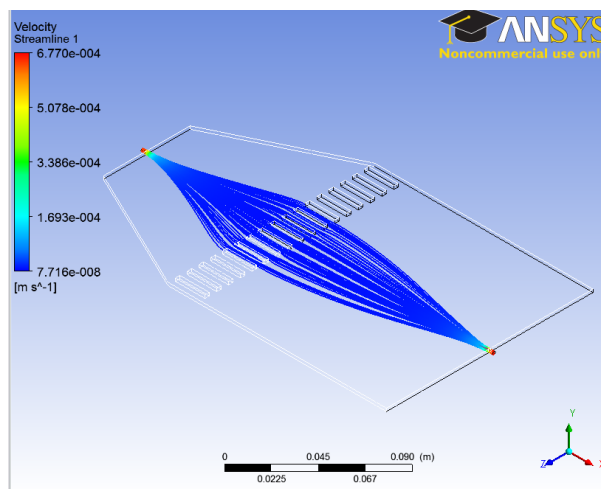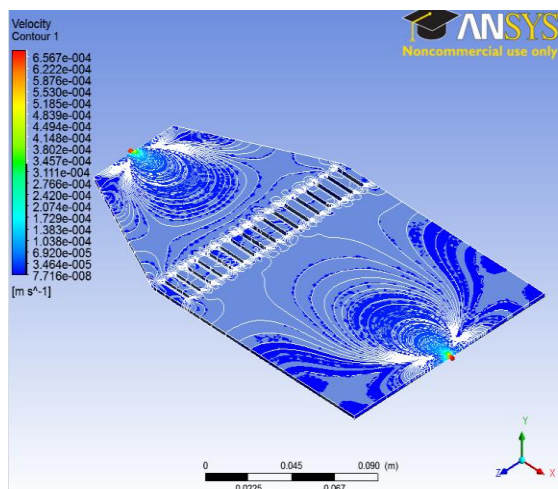
Appendix D: CFX Analysis – Contours and Streamlines
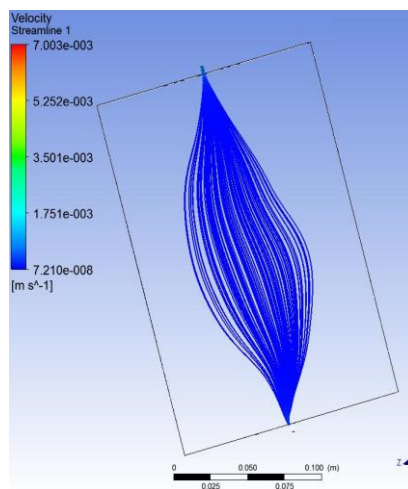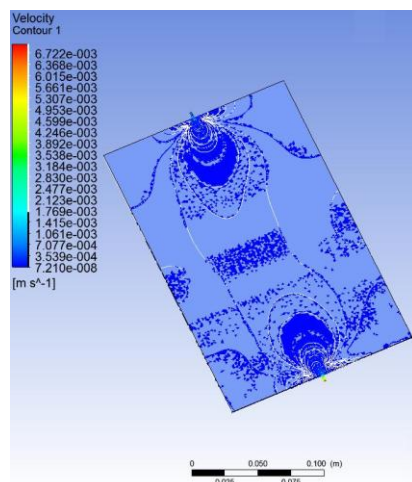
The velocity contours for the rectangle geometry are on the left and 100 streamlines through the cassette are on the right, determined using computational fluid dynamics (with Ansys CFX software). The boundary conditions used were: no-slip walls, inlet velocity of 0.76 mm/s, and outlet pressure of 0 Pa. The dimensions of the figure can be seen in Appendix C.
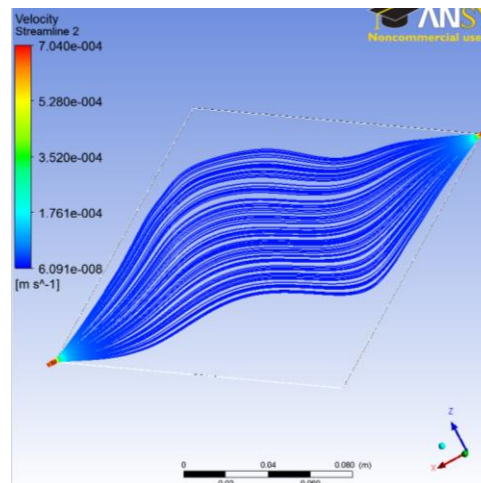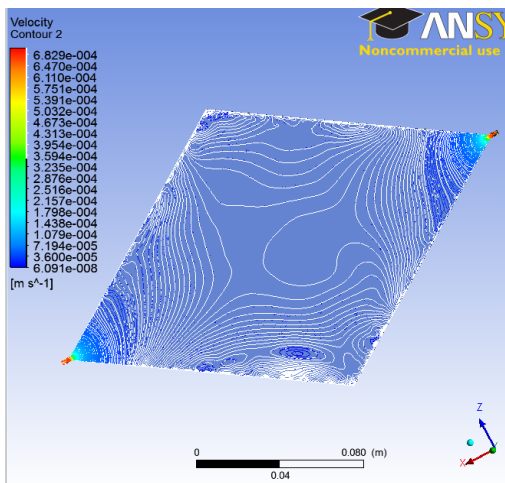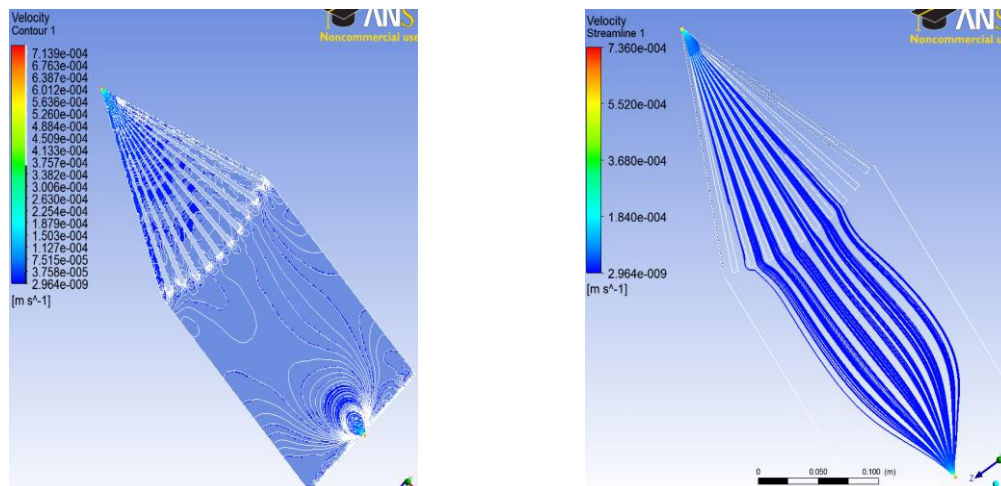

The velocity contours for the diamond geometry are on the left and 100 streamlines through the cassette are on the right, determined using computational fluid dynamics (with Ansys CFX software). The boundary conditions used were: no-slip walls, inlet velocity of 0.68 mm/s, and outlet pressure of 0 Pa. The dimensions of the figure can be seen in Appendix C.

The velocity contours for the ellipse geometry determined using computational fluid dynamics (with Ansys CFX software). The boundary conditions used were: no-slip walls, inlet velocity of 0.44 mm/s, and outlet pressure of 0 Pa. The dimensions of the figure can be seen in Appendix C.

The velocity contours for the fan with straighteners geometry determined using computational fluid dynamics (with Ansys CFX software). The boundary conditions used were: no-slip walls, inlet velocity of 0.67 mm/s, and outlet pressure of 0 Pa. The dimensions of the figure can be seen in Appendix C.

The velocity contours for the guided geometry are on the left and 100 streamlines through the cassette are on the right, determined using computational fluid dynamics (with Ansys CFX software). The boundary conditions used were: no-slip walls, inlet velocity of 0.67 mm/s, and outlet pressure of 0 Pa. The dimensions of the figure can be seen in Appendix C.
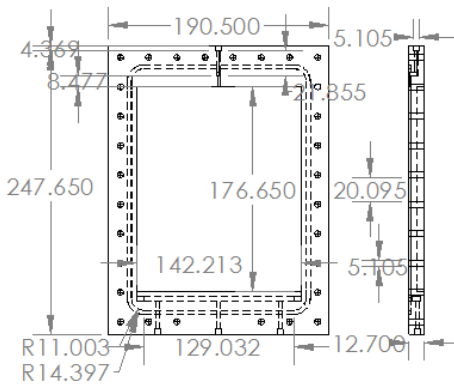
Appendix E: Prototype Dimensions
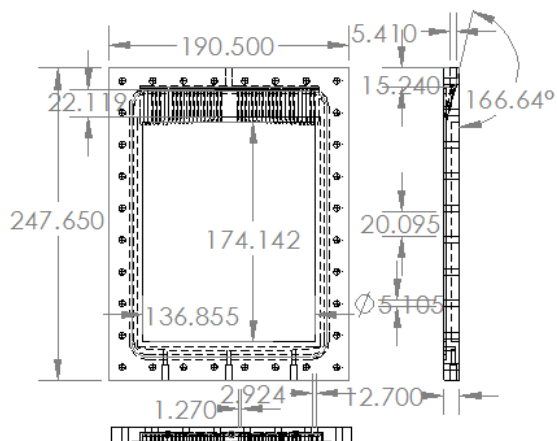
Note: All dimensions are in mm.
Rectangle:
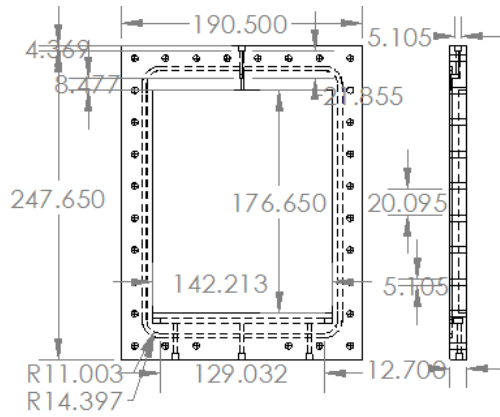
Balanced Runner:

Straws:

Appendix F: Bubble Trap Dimensions

Note: All dimensions are in mm.