Progress Toward the Design of a Perfusion Bioreactor Cassette for Stem Cell Growth

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Abstract

Stem cells show great potential for use as patient-specific medical therapeutics. In order for this therapy to be effective, a bioreactor cassette system is required that is capable of providing conditions for growing multiple stem cell samples from individual patients without exchanging media between samples. Such a cassette must also be gas impermeable, able to be visualized on a standard microscope, and able to deliver evenly distributed fluid flow over its cell growth area. Problems with bubble removal and human factors quickly became evident in the first design iteration, so it was revised with these considerations in mind. A single-use cassette with optimized fluid flow distribution, live-cell imaging capabilities, minimized material use, and improved ergonomic qualities was designed. These properties were confirmed by dye studies, preliminary cell seeding studies, cost analysis, and ergonomic testing. This device is near completion and ready for mass production using injection molding and ultrasonic welding, but studies still need to be undertaken to confirm scale-up possibilities and cell growth during perfusion.

Introduction

Stem cells, through which any of the body's approximately 200 cell types can be 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 (Ling Yi & Lin 2009). Stem cell-based therapeutics, or treatments in which induced differentiation of stem cells is used to repair or replace damaged cells or tissues, are showing great progress in their potential for use in a clinical setting (Ling Yi & Lin 2009). Until recently, stem cells have been used primarily to model cell growth and perform drug screening. As the potential for implantation and use in a patient approaches, however, researchers need a way to grow stem cells under individually controlled conditions, without daily maintenance. Stem cells are notoriously difficult to culture. They are heavily influenced to remain pluripotent or to differentiate depending on the growth factors, neighboring cells, and waste products in the media. For example, constant exposure of stem cells to bFGF, TGF-β1, and insulin helps them to remain pluripotent (Ludwig *et al.* 2006). Being close to other neighboring cells near confluence exposes a cell to other cells' secreted factors, which can induce differentiation. Waste products such as lactate and ammonia are known to primarily induce cell death (Patel *et al.* 2000). Learning to culture stem cells can take months to years of careful instruction to interpret the cells' state accurately (Ulloa-Montoya, Verfaillie, and Hu 2005). Currently, researchers working with many stem cells are required to change the media every day, making culture tedious and time-consuming (Ouyang, Ng, and Yang 2007). These specific needs are well suited for automated stem cell growth using a bioreactor. A perfusion bioreactor system with specialized culture chambers called "bioreactor cassettes" has the potential to replace the current static culture standard. Though several devices exist for either static culture or perfusion bioreactors, none are able to evenly control environmental factors to specifically guide directed pluripotent growth or differentiation in stem cells.

Bioreactor cassettes are small, specialized culture dishes that can be used to grow stem cells (Shi and Clegg 2008). They can be attached in a network to a bioreactor (Figure S1), which incubates and provides them with a continued supply of media containing appropriate growth factors. Once a sufficient number of cells have grown, they can be subjected to specific chemical or mechanical signals that direct their differentiation into a desired cell type. It is essential for a perfusion cassette to facilitate even fluid flow over the cells. Dead spaces in media flow can result in accumulation of metabolic waste or

insufficient exposure to pluripotency and growth factors, causing cells to differentiate or die. A homogeneous cell population will be important for therapeutic purposes, but recirculation currents in the cassette could induce cell-cell signaling pathways, leading to differentiation (King and Miller 2007). Additionally, researchers using the current gold-standard of static culture flasks expect to be able to visualize their cells using a microscope at any point in the growth process. In order to make stem cell therapy accessible on a large scale, it will be necessary to have an economically and ergonomically viable cell culture cassette to interface with a bioreactor system. Here, we present a device that meets these expectations.

Preliminary Design

The design requirements associated with the device ensure its ability to produce a final homogeneous population of stem cells. First, the cassette must facilitate confluent healthy stem cell adherence and growth by encouraging even fluid flow coverage over the entire cell growth area. Bubbles should be prevented from entering the cassette, and any residual bubbles that accumulate within the cassette due to media off-gassing should be easily removed. The cassette must interface well with a standard inverted microscope for imaging while remaining connected to the bioreactor tubing. Priming and loading of cells must be easily done in a sterile and straightforward manner within the confines of a biological safety cabinet.

The first realization of these requirements resulted in a large-scale device, nearly 25x19x1 cm with 238 cm^2 growth area, in which a balanced channel design was used to guide fluid flow (Figures 1, S2). The flow was evenly divided three times before reaching the cells, and then collected in the same fashion at the outlet using a channel design mirroring the inlet. To facilitate testing of the initial prototype, the device was designed to interface with a pre-fabricated tissue culture treated piece of polystyrene and a heavy piece of lexan plastic for added stability and ease of screw connections. A thin silicone gasket material was used to cover the inlet and outlet channels as well prevent leaking. The polystyrene and lexan plates were placed on top of the silicone gasket and secured to the frame using 28 screws. The entire assembly process took over 45 minutes to complete, and leaking occurred frequently due to improper tightening of screws and gasket rippling. The difficulty of dealing with bubbles in this cassette required the design of an external bubble trap to remove air bubbles from inlet media.

The bubble trap design placed the inlet port higher than the outlet because bubbles in the perfusion system are less dense than the media. In the 3 x 4 x 3.4 cm box, the bubbles moved upward in the system and collected in the top of the trap. The trap was split into two chambers using a diagonal screen (Figures 2, S3). Bubble passage was limited by size selection by the incorporation of 2.85 mm diameter holes in the lower portion of the screen. The holes have collective larger cross-sectional area than the outlet in order to avoid possible pressure build-up.

Due to many undesirable characteristics of the first prototype (Prototype A) design including the difficulty of removing bubbles, the difficulty of imaging, and user orientation confusion (*vide infra*, Human Factors Study), a second prototype (Prototype B) was designed (Figures 3, S4, S5). The overall size, approximately 14x9x2 cm with 99 cm^2 cell growth area, matches the dimensions of a 96-well cell culture plate which fits well on a typical inverted light microscope stage. Instead of a balanced channel system, the flow division mechanism consists of 17 size-scaled straws in which the diameter of the straw and distance from the inlet was approximated using a simplified Hagen-Poiseuille equation. In this approximation, the length the media traveled from the inlet port to the cell growth area was proportional to the radii of the straws raised to the fourth power. The 3 outlet channels, reduced for simplicity, were scaled using the same principle. The polystyrene plate was secured to the prototype using a silicone adhesive in order to remove the necessity of a gasket and screw connection system. The final mass

production of the device, however, would result in a single continuous piece, obviating the need for an external adhesion mechanism. This design incorporated a separate bubble removal port connectable to a syringe to aid in cassette priming as well as air removal resulting from media off-gassing during a perfusion run. The volume of material used to prototype this cassette is 2.1-fold less than for Prototype A, after normalizing for cell growth area.

Materials and Methods

Cassettes

All iterations of the bioreactor cassette design were modeled in SolidWorks prior to fabrication. Early versions (Prototype A) were fabricated with polycarbonate using the Stereolithography Apparatus or machining capabilities at the Rapid Prototyping Center at the Milwaukee School of Engineering, under the direction of Dr. S. Kamara. Later versions (Prototype B) were fabricated with Accura60 using the Stereolithography Apparatus in the Wisconsin Institute of Discovery at the University of Wisconsin–Madison, under the direction of Dr. T. Mackie.

Cassette iterations were fabricated as a frame, and assembled into a complete cassette using polystyrene plates cut to the desired size from Corning® culture flasks (Figure 4). In early versions (~238 cm^2 growth area), the polystyrene plate was temporarily secured to the prototyped frame using a silicone gasket and 28 screws, evenly spaced around the cell growth area. The frame was re-used. For Prototype B, the polystyrene plate was secured to the prototyped frame using permanent silicone adhesive and Scotch liquid superglue. The frame was not re-used.

Bubble Trap

The bubble trap was fabricated in two parts from Accura60 using the Stereolithography Apparatus in the Wisconsin Institute of Discovery at the University of Wisconsin–Madison. The top piece was permanently attached using Devcon Epoxy Plastic Welder.

Connections

Devices were connected to tubing using 1/4" 28 threaded polypropylene luer fittings from McMaster Carr and stainless steel 1/16" NPT luer fittings from S4J Manufacturing Services. DAP Aquarium Sealant 100% Silicone (Clear) was purchased to coat connection points on later devices in addition to SuperGlue. PharMed BPT 0.89 mm tubing was used to facilitate perfusion.

Fluid Flow Profile

Dye studies were performed to obtain fluid flow profiles of each cassette iteration. The assembled cassette was primed with distilled water and perfused, at a rate of 8 volume exchanges per day, by a Masterflex EW-07519-20 peristaltic pump. Distilled water with green food coloring was used as the fluid. Over the course of the study, images were obtained once each minute using a PlantCam (Wingscapes) time lapse camera supplemented with neutral density filters for at least 3 hours. An LED light box was place under the cassette during recording to improve contrast. Results were evaluated qualitatively.

Imaging and Cell Seeding Density Study

Attempts to visualize cells in each prototype and specifically analyze cell seeding density were performed on Prototype B (as Prototype A was unable to be imaged without disassembling the cassette). HEK 293AD cells were seeded into Prototype B and allowed to adhere for 3 hours. Complete media (DMEM, 10% FBS, 2.5 mM Glutamax, Antibiotic & Antimycotic) was then gassed with a biological atmosphere of a mixture of hydrogen, 9.4% carbon dioxide, nitrogen, and 7.9% oxygen. Cells were imaged through the cassette frame at 9 representative areas near the inlet, center, and outlet of the cassette. The cells were counted in each of the images and the numbers were extrapolated to find an average density per section. Cell density was analyzed using a 2-way ANOVA (95% confidence interval) to determine if there were differences between cell density near the inlet, middle, and outlet of the cassette.

Human Factors Study

Compatibility of each cassette (Prototypes A and B) with human users was evaluated. Nine subjects with cell culture experience were selected to participate in a testing and survey combination. Each participant was given instructions, asked to prime both prototypes separately, and asked a series of questions about ease of manipulation, orientation, and priming for each cassette (Survey S1). Survey proctors monitored progress and evaluated success of each participant by rating the priming success on a scale of 1-10 (with 10 being perfect priming with no bubbles or air left in the respective cassette). The moderator also recorded the time that each participant spent priming each cassette. Data was analyzed using a two-tailed Student's t-test (95% confidence interval) where appropriate.

Bubble Trap Efficiency Study

In order to test the ability of the bubble trap to remove bubbles, the bubble trap was first primed completely with media using clear tubing at both the inlet and outlet. A syringe filled with media and air was then connected to the inlet tubing. To form bubbles (with a volume of approximately 70 µL, based on observed diameter of about 5 mm), the syringe was tipped, allowing air to enter the inlet tubing. The number of bubbles entering and exiting the trap was counted in order to calculate the bubble trap's efficiency. Efficiency (E) was defined as the following:

$$E = \frac{\left(bubbles_{enter}\right) - \left(bubbles_{exit}\right)}{\left(bubbles_{enter}\right) + \left(bubbles_{exit}\right)} \times 100\%.$$

Results

Fluid Flow Profile

The results of the dye studies suggest that Prototype B has flow than Prototype A (Figures 5,6). Prototype A seems to start out fairly even, but a parabolic flow pattern with more exchange in the middle becomes evident after approximately 10% of the cassette has been perfused, and the outlet region leaves dead spaces near the outside edges (Figure 5). Additionally, this design was heavily affected by a large bubble formation occluding the collection of fluid from the outlet region. Prototype B, on the other hand, has consistently even flow until near the outlet, where the edges receive less fluid exchange (Figure 6). However, it is possible that the large bubble occluding the flow in the upper corner of the outlet region may have contributed to the slight imperfections in outlet flow.

Imaging and Cell Seeding Density Study

The results of our cell seeding and imaging study were encouraging. First and foremost, we found that it is easy to image the cells in Prototype B using an inverted light microscope, though the striations in

the rapid prototyped material of the cassette are also seen in the images (Figure 7). The cell densities in each section were compared using a 2-factor ANOVA without replication and no significant difference between the three sections was found (Figure 8). However, there was a trend toward decreasing cell density from the inlet to the outlet (despite no statistical significance).

Human Factors Study

The results of the human factors study supported the improvement of Prototype B compared to Prototype A . Analyzing the time and success of priming for each participant for each cassette revealed significant differences between the two cassettes (Figure 9). The average time spent priming Prototype A was 7 ± 2 minutes while the average time spent priming Prototype B was 3 ± 1 minutes (2-tailed t-test, $p<0.05$). The average priming rating that the moderator awarded Prototype A was 6.7 ± 1.3 while the average rating for Prototype B was 9.3 ± 0.5 (2-tailed t-test, $p<0.05$). The ratings that the participants gave the ease of priming each cassette were also significantly different between prototypes (Figure 10). The average rating, out of 5, for Prototype A was 1.4 ± 0.5, while the average rating for Prototype B was 3.7 ± 0.5 (2-tailed t-test, $p<0.05$). Likewise the user-rated ease of priming each cassette in a biological safety cabinet was also significantly different between prototypes, with Prototype A receiving an average of 1.3 ± 0.5 and Prototype B receiving an average of 3.2 ± 0.7 (2-tailed t-test, $p<0.05$) (Figure 10). Additionally, all nine users were able to correctly choose the inlet and flow direction for Prototype B. Finally, while all nine users were able to correctly choose the orientation of Prototype B, only four were able to correctly choose the orientation of Prototype A (Figure 11).

Bubble Trap Efficiency Study

Our efficiency test revealed that of 50 bubbles injected, no bubbles were detected leaving the outlet of the trap, giving it an efficiency of 100%. In this test, 50 bubbles were not enough to overwhelm the trap; however, if the total bubble or air volume exceeded 15 mL, bubbles entered the outlet tubing. To increase this overwhelm volume, modifications to the trap could include increasing the volume of the upper chamber of the bubble trap.

Discussion

As can be seen from the dye study results, the size-scaled flow tubes design offers an exceptionally evenly distributed flow profile, as compared to the previous inlet design (Prototype A). This is important in the automated culture of stem cells, as dead spots or eddies in flow would create inconsistencies in the environment in the bioreactor cassette. Build-up and recirculation of waste factors in some areas of the cell growth area could prevent optimal growth of the stem cells, or premature, uncontrolled differentiation from the pluripotent state (Artmann, 2011). Differences in the shear or mechanical forces applied to cells in different regions of the cassette could cause similar effects (Fong, 2007). Results of the cell seeding procedure with Prototype B indicate that the inlet straw flow guides evenly distribute the cells across the growth area of the cassette. Even distribution has been shown to improve growth characteristics and yield of cells with time, as concentrated clumps of cells excrete waste which can hinder cell growth (Lanza, 2004). It has also been shown, however, that autocrine interaction is necessary for successful stem cell growth, especially initially after seeding. Future work could confirm the ideal seeding density for growth based on level of autocrine interaction necessary for optimal growth.

Imaging tests indicate that cell growth observation is possible and successful with Prototype B. Imaging protocol likely disturbs the cell growth environment no more than in currently used static culture methods, and yields images of comparable quality. “Scratches” observed in the current images are a

remnant of the rapid prototyping procedure used to build these early iterations; fabrication of cassettes using injection molding of polystyrene will likely eliminate this effect and further improve image quality, making all cell assays traditionally used in static culture possible in the new bioreactor cassette. Also, design of the cassette in the standard 96-well footprint makes it compatible with existing imaging equipment commonly used in target market facilities and laboratories. Future work will involve performing select traditional assays—trypan blue, crystal violet, and immunostaining—to confirm these assumptions.

The cassette targets 16% ($13.63 billion) of the total scientific research and development market in medical sciences and 57% ($52.67 billion) of human health technologies of the biotechnology industry, and seeks to dramatically reduce their cost of operation and rate of error and contamination by automating stem cell growth (Kaczanowska 2011; Snyder 2011). Correct, easy-to-understand use of the bioreactor cassette is therefore of primary concern. Preliminary ergonomic studies for Prototype B are promising. Our testing and survey studies show that the time required to prime the bioreactor cassette for a user with cell culture experience but without practice with our cassettes is approximately 3 minutes, which minimizes the time that the stem cells will be held in sub-optimal growth conditions (at 23° C, instead of 37°C). Furthermore, the ability and ease with which users can remove bubbles, which might occlude flow and cause uneven media distribution or dead spots within the cell growth area, is greatly improved in the most recent iteration of the bioreactor cassette, Prototype B (Figure 9). We suspect that this is because of the addition of a sloped bubble port over the cell growth area, as well as fewer bends in the straw inlet design, compared to Prototype A, even though the diameters associated with the inlet of Prototype B are smaller than the diameters of the flow guides in Prototype A. We suspect that ease and frequency of bubble removal will only increase as this new cassette iteration is scaled up to a growth area comparable to that present in Prototype A.

Further human factors testing indicates that users are highly likely to orient Prototype B correctly (Figure 11) This is important, as incorrect orientation can result in failed cell growth, which would be costly and wasteful for the user (cell culturist) and inconvenient for the patient benefiting from the stem-cell therapy, as a return visit and second biopsy might be necessary as a result.

Finally, when cassette users were asked to choose between the older and newer iterations of the bioreactor cassette (Prototypes A and B, respectively), all nine participants believed that the newest iteration was easiest to prime, and would be easiest to use in a sterile hood. This indicates that human factors were not sacrificed for the previously discussed improvements to the ease of microscopy and flow characteristics of the bioreactor cassette. Future work might confirm these study results with increased sample size.

In the bioreactor system, media is held at 4°C before it is pumped to be oxygenated, and then warmed to 37°C before it is perfused into the bioreactor cassette. It has been shown that changes in temperature can affect gas solubility (Fogg & Gerrard, 1991) . The smaller cassette design decreases total media holding volume in the cell growth area of the cassette, increasing the percentage of area affected by an air bubble reaching the body of the cassette. Because stem cells require a carefully controlled environment for specific growth, and despite the fact that the material forming the cassette is gas impermeable to avoid diffusion of gas into the external environment, this potential phase separation could prevent appropriate growth. Further testing should be done to determine if and at what holding volume this low-level off-gassing affects stem cell growth properties.

Material use in the redesigned cassette, Prototype B (normalized for cell growth area) , is 2.1 fold lower than that for Prototype A. Not only will this reduce cost associated with cassette manufacture, it will also reduce waste. Polystyrene takes up more space in landfills than paper, takes centuries to deteriorate, and energy consumption in production also significantly impacts the environment (Tabone *et al*, 2010). This represents significant benefit to the environment, as an estimated 550,000 cassettes will be sold per year, and the material choice is necessary to accommodate cell growth and high standards of patient safety.

Aside from making removal of de-gassed bubbles possible during a perfusion run without damaging the cell growth environment and while maintaining sterility, the addition of a bubble port over the cell growth area can double as a quick port from which to deliver solutions to the cell growth area. This is especially useful if the delivery volume is less than the cassette hold-up volume. For example, solutions for routine treatments such as trypsinization or antibody staining can be quickly added via syringe or pipette through the standard luer port which comprises the bubble port, and evenly distributed over the growth area with gentle shaking of the cassette body.

Conclusions

Overall, we present two functional perfusion bioreactor cassette prototypes. Prototype A has a larger cell growth area and the flow is oriented through channels that split equally in half three times before directing the fluid over the cell growth area. The outlet involves a mirrored inlet. However, problems with bubble removal, user-friendliness, and imaging prompted the design of another smaller prototype, Prototype B. Prototype B was designed to fit a 96-well plate footprint and to be more understandable to users while allowing imaging and ease of priming and bubble removal. The inlet splits the flow using 17 size-scaled channels, which ultimately result in a more even flow distribution as indicated by dye study results. Additionally, Prototype B can be seeded evenly from inlet to outlet as indicated by a cell seeding study. Participant survey studies indicate that all nine participants (with cell culture experience) found Prototype B easier and faster to prime and remove bubbles from than Prototype A. These improvements make Prototype B an excellent candidate for use in automated, perfused bioreactor systems as clinical stem cell therapies become a reality.

Acknowledgements

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Nomenclature

Pluripotent: The ability of a stem cell to differentiate into any cell type of the body from one of three germ layers: endoderm, mesoderm, and ectoderm.

Perfusion: the pumping of fluid to deliver nutrients and specific factors to cells.

Bioreactor cassette: Cell containing enclosure intended to connect with a perfusion bioreactor, which automates the flow and nutrients cells should receive.

Priming: Completely filling the cassette with fluid.

Supplemental Information Available: Supplemental information are reported herein.

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Figures

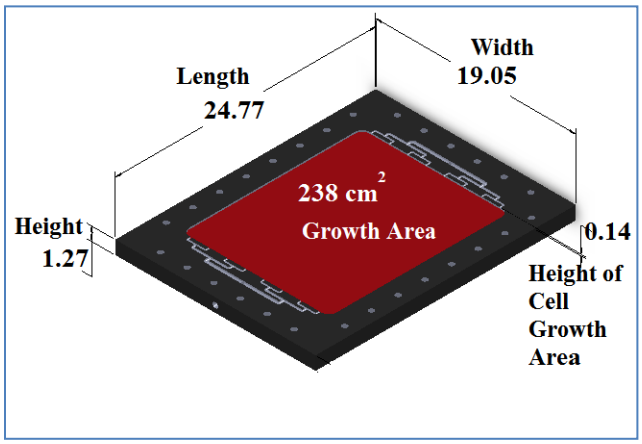


Figure 1: Prototype A with larger area for cell growth and balanced channels for even flow division and collection

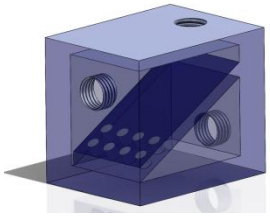


Figure 2: Bubble trap to allow bubbles to collect in top chamber while preventing their passage through a diagonal screen.

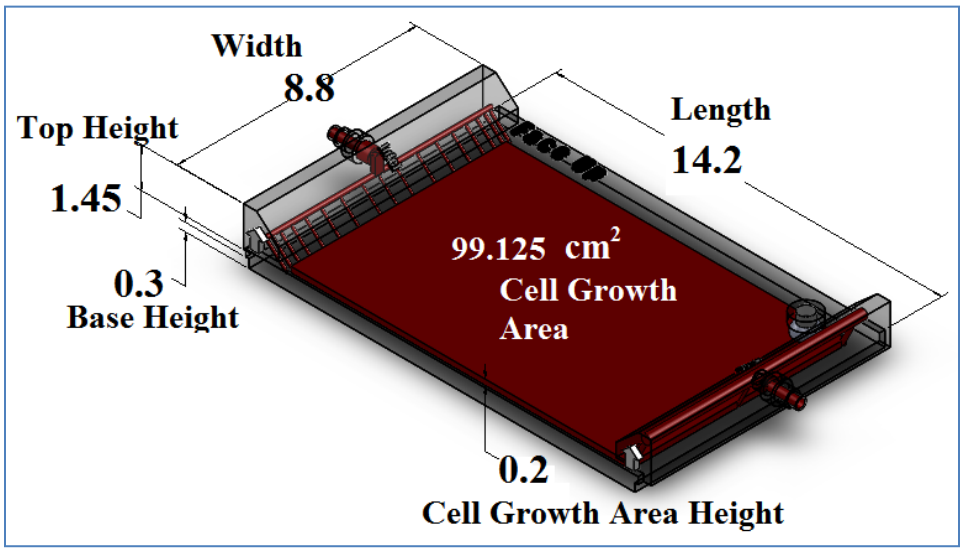


Figure 3: Prototype B with smaller cell growth area designed specifically for more even fluid flow, cell imaging, and human factors

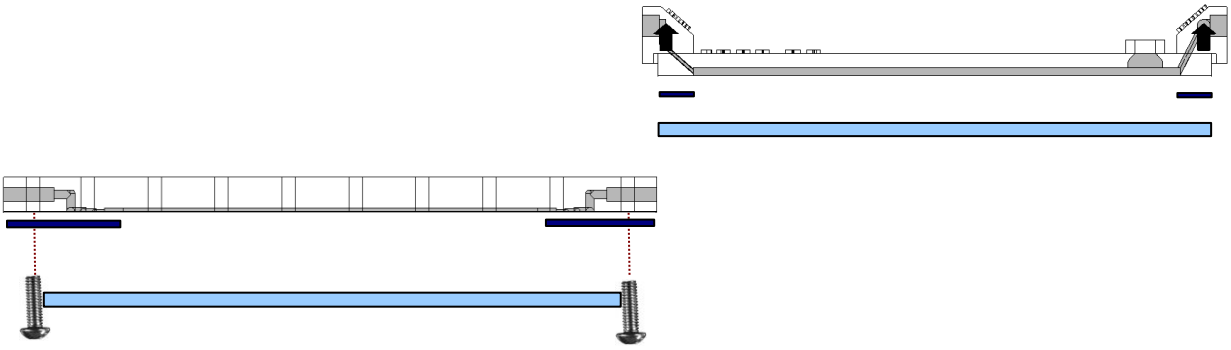


Figure 4: Prototypes A and B setup involves attaching a polystyrene plate (blue rectangle) to the cassette frame.

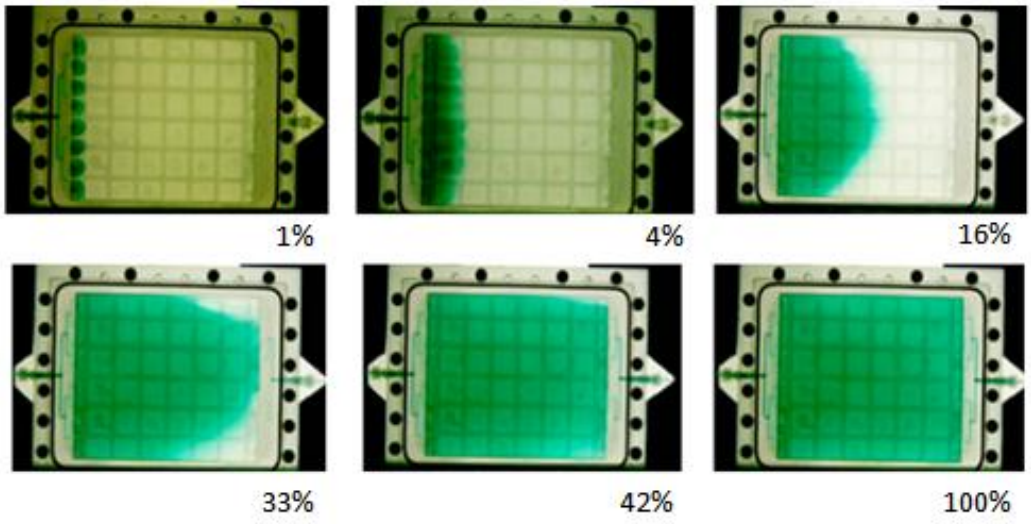


Figure 5: Dye study of Prototype A with images selected from various points in time to give a complete picture of the results.

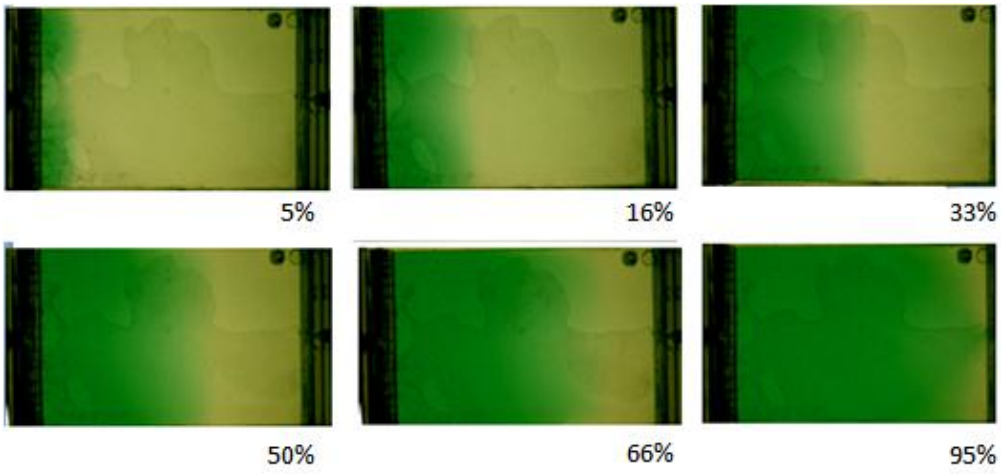


Figure 6: Dye study of Prototype B with images selected from various points in time to give a complete picture of the results.

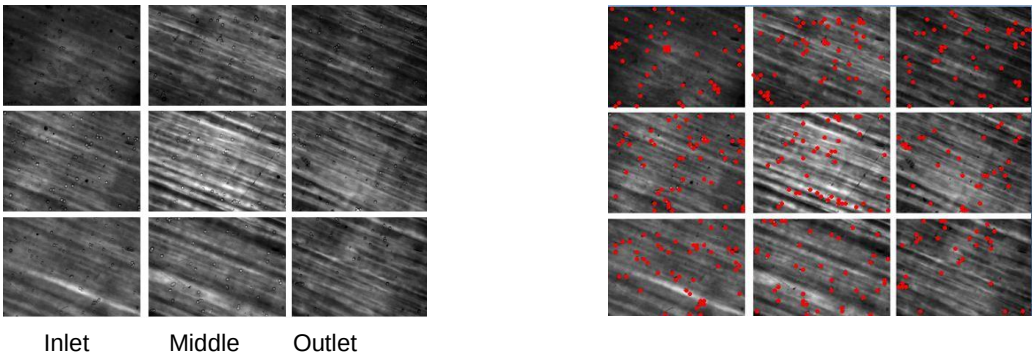


Figure 7: Cell images using phase contrast with an inverted light microscope. Images on the right are the same as on the left, but with the cells colored in red for easier viewing.

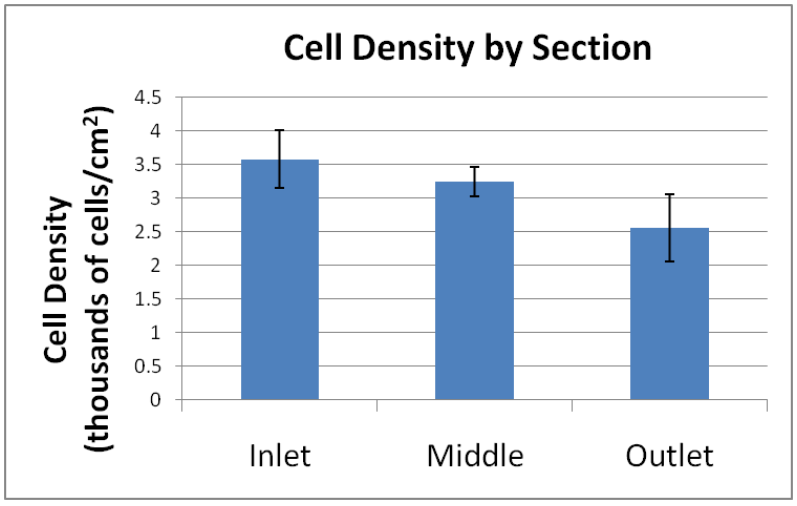


Figure 8: There was no significant cell density difference between the regions at the inlet, middle, and outlet of Prototype B ($F(2,2 = 6.94, p=0.08$). Regions are labeled in Figure 7.

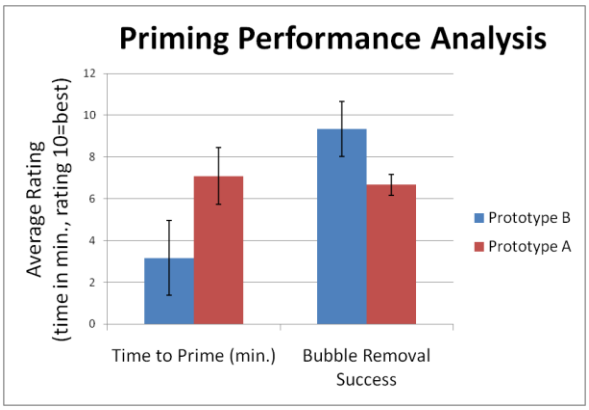


Figure 9: Time spent priming and bubble removal success (10=complete removal) of each prototype (n=9; error bars represent ±1σ; Student's t-test p<0.05 for each category).

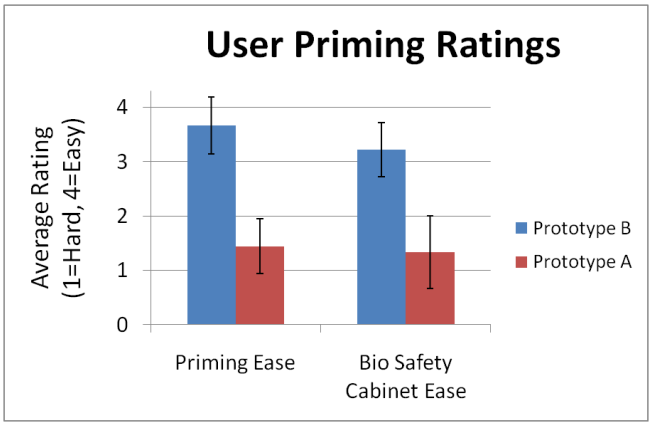


Figure 10: User-rated priming ease and practicality of priming each prototype in a biological safety cabinet (n=9; error bars represent ±1σ; Student's t-test p<0.05 for both categories).

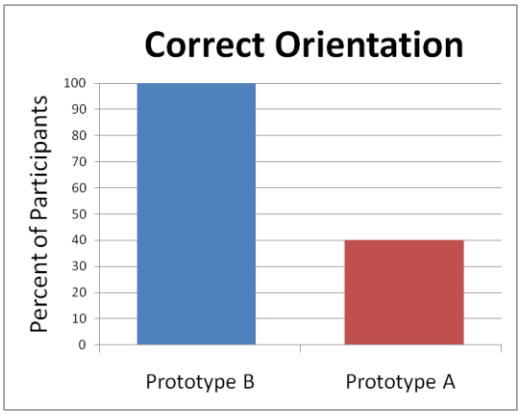


Figure 11: Percent of participants who correctly oriented Prototype A and B after priming (n=9).

Supplemental Information

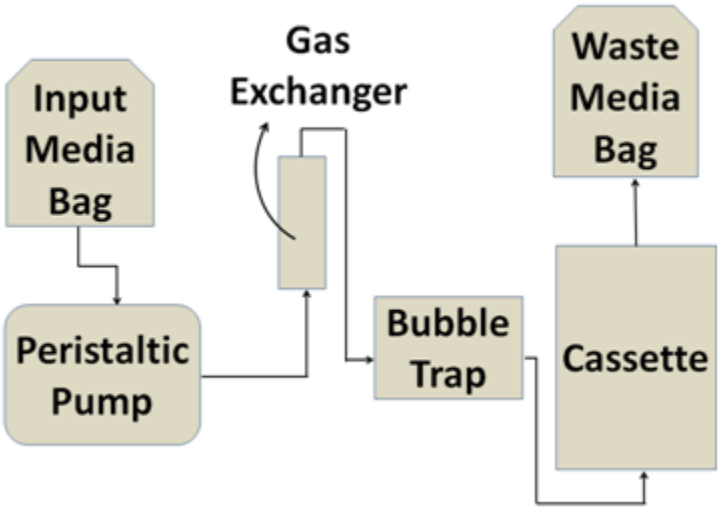


Figure S1: The bioreactor system setup is shown here with arrows indicating the direction of media flow.

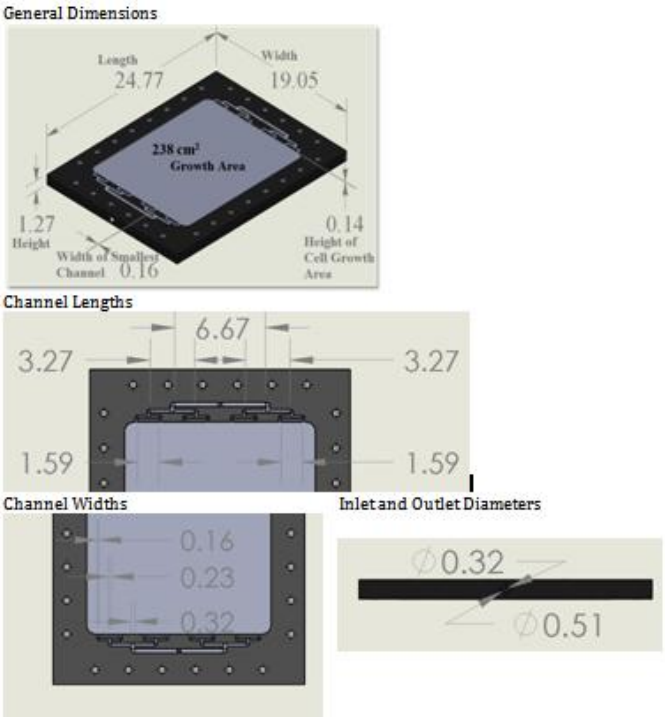


Figure S2: Prototype A Dimensions (in cm)

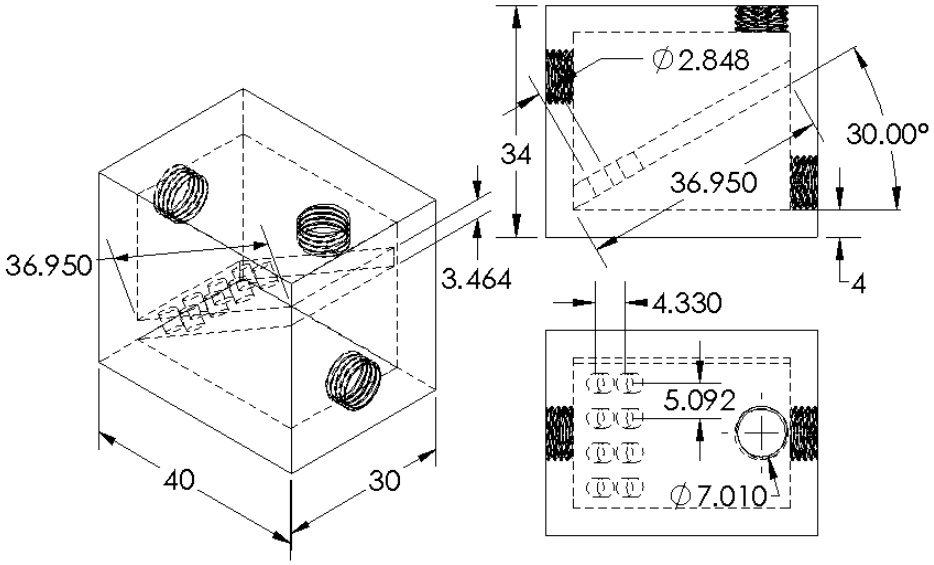
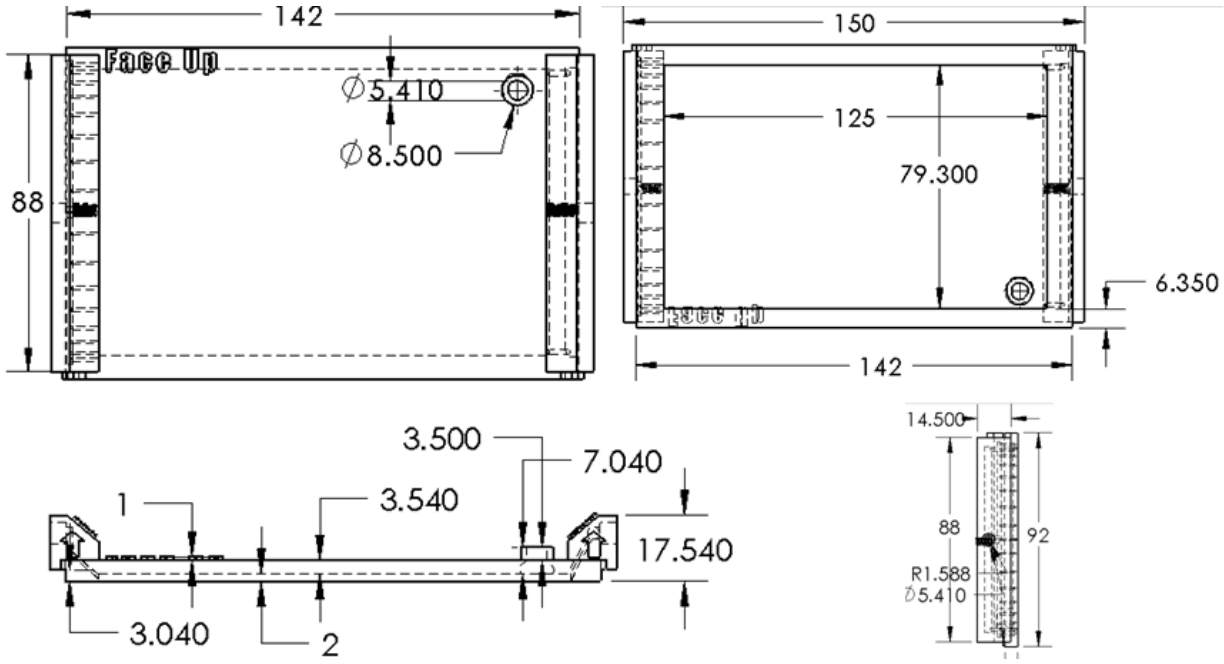


Figure S3: The dimensions and design of the external bubble trap are given in mm here.



Straw dimensions at inlet and outlet

Figure S4: Prototype B Dimensions (in mm).

Straw #	d from center (mm)	radius (mm)
1	41	0.95
2	39	0.94
3	36	0.92
4	32	0.90
5	27	0.87
6	21	0.83
7	14	0.78
8	6	0.70
9	0	0.62

Figure S5: Prototype B inlet channel design. Straws 1–8 are mirrored on both sides of the center straw (9). The distance from center indicates the distance from the center of the middle straw (9) to the center of the respective straw.

Survey S1:

Ergonomic Survey Instructions and Questions

Bioreactor Cassette Ergonomics Survey

All responses will be kept anonymous. Data will be presented as bulk trend only; comments may be anonymously used to explain bulk trends

Part 1: Priming and Bubble Removal Procedure

Priming = filling the cassette with fluid and removing as much air or as many bubbles as possible. A well-primed cassette would be completely full of fluid and have no air bubbles.

Please follow the given instructions to prime each cassette. Then rate and describe your experience with each cassette on the following survey.

Priming Instructions for the Smaller Cassette:

1. Please ask to be shown how to use a three-way valve to assist you in this setup.
2. You will be given a syringe filled with water.
3. Screw the syringe into the luer fitting on the three way valve connected to the tubing at the **INLET.**
4. Open the three-way valves at the **INLET** and the **OUTLET**
5. Push the syringe plunger in to fill the cassette with fluid. It may be easiest to fill the cassette by holding vertically and manipulating the position of the cassette while filling.
6. Tap the cassette to coalesce any residual bubbles, and position the bubble under the **BUBBLE PORT** (small protrusion located on top of the "Face Up" side).
7. Close the three-way valve at the **OUTLET** and remove the cap from the **BUBBLE PORT.**
8. Inject more fluid through the **INLET** to push the bubble out of the **BUBBLE PORT**.
9. Replace the cap on the **BUBBLE PORT** and close the three-way valve at the **INLET.**
10. Unscrew the syringe from the three-way valve.
11. Please give the filled cassette to a facilitator to examine.

Priming Instructions for the Larger Cassette:

1. Screw the syringe into the luer fitting on the three way valve connected to the tubing on one side
2. Make sure three-way valves are open on both sides.
3. Push the syringe plunger in to fill the cassette with fluid. It may be easiest to fill the cassette by holding vertically and manipulating the position of the cassette while filling.
4. When full, to remove residual air/bubbles- vigorously tap the cassette then inject fluid to push bubbles through the outlet. Refill syringe if necessary, making sure to close three-way valve before removing from luer.
5. Close both three-way valves.
6. Unscrew the syringe from the three-way valve.
7. Please give the filled cassette to a facilitator to examine.

Please use the following scale (1-5) to rate your experience with each cassette.

1=Very Difficult, 2=Moderately Difficult, 3=Moderately Easy, 4=Very Easy,

How easy was it to prime the cassette, including removing the air/bubbles?

	Hard			Easy
Smaller Cassette	1	2	3	4
Larger Cassette	1	2	3	4

How easy would it be to remove bubbles from this cassette in a biological safety cabinet?

	Hard			Easy
Smaller Cassette	1	2	3	4
Larger Cassette	1	2	3	4

Please State:
Which cassette was easiest to prime (circle one)?

Smaller **Larger**

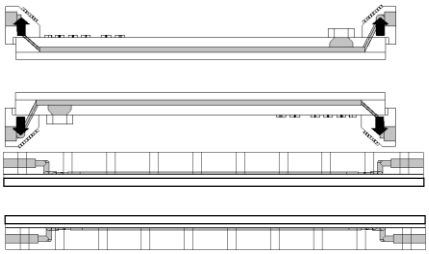
Why?

Part 2: Orientation Questions

Carefully physically examine each cassette that you have just primed.

Which orientation is correct for the intent of growing cells connected to a bioreactor in an incubator? (If the pictures are unclear, ask to be shown each orientation with the cassettes)

Circle one of the pictures below from each category, smaller cassette and larger cassette.



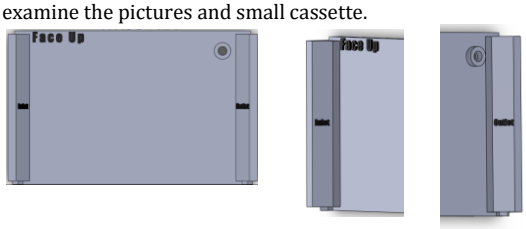
How confident are you that the orientations you circled above are correct?

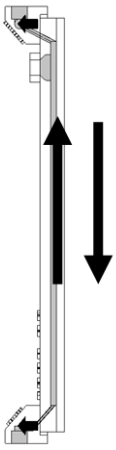
	Not Sure				Confident
Smaller Cassette	1	2	3	4	5
Larger Cassette	1	2	3	4	5

Which cassette orientation was easiest to determine?

Smaller **Larger**

These cassettes hook up to continuous flow at the inlet, which perfuses through the body of the cassette, and flows out the outlet. Please examine the pictures and small cassette.





Please circle one of the arrows to the right of the side-view of the small cassette to indicate the direction of fluid flow.

<u>Part 3: Questions asked by facilitator</u>
(don't need to give these to the test subjects):

- Record success of priming each prototype (10=perfect, no bubbles, 1=worst rating)
- About how long did it take to prime each cassette (numbers in minutes)?

Smaller Cassette	0.5	1	2	3	4	5
Larger Cassette	0.5	1	2	3	4	5