Microscope Compatible Cell-Culture Incubator

BME 400
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Abstract

Live cell imaging experiments are difficult to perform over long periods of time on normal lab microscopes without incubation capabilities. Current commercial microscope-stage incubators are expensive, not compatible with multiple microscopes and ineffective at evenly controlling the environment. The client desires an inexpensive incubation chamber compatible with cell microscopy that is capable of maintaining desired temperature, CO_2, and humidity evenly throughout the chamber. The device should not alter image quality, and should be accessible for changing media or cell culture dishes. An initial prototype has been developed that involves a small, cohesive system to regulate temperature, CO_2, and humidity. Testing of the current prototype has demonstrated regulation of these three systems, through an automated feedback system capable of maintaining the system near a physiological set point. Further development of the design will ensure that it performs efficiently according to all specifications, and ultimately help bridge the gap in the market between high-cost, functional incubation systems and cheaper, less effective designs.

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Introduction

Motivation

Many experiments in cell biology and tissue engineering require the observation of transient or long term cellular response to a treatment of interest. Removal of cells from incubators during these experiments can introduce sources of experimental error, in addition to being inconvenient. The ability to image and incubate cells simultaneously eliminates this error and is recognized as a valuable tool to researchers. After talking with campus researchers, it appears that current microscope incubation systems are often unreliable in maintaining environmental stability, or are constructed by integrating a microscope into an incubator, limiting the user to imaging modalities of a single scope and driving up cost. The client has identified a gap in the current market for an affordable incubation system capable of sitting on the stage of a microscope stage.

Existing Market

The current market for microscope incubators exists in two distinct classes. The first class is comprised of high end systems whose costs reflect the combination of high end automated optics, integrated within a custom incubator system. One such product, the Nikon BioStation IM-Q, houses an entirely automated imaging system in its incubation chamber, seen in Figure 1 [1]. While the system allows for a high level of environmental control, it is only compatible with the microscope enclosed in the chamber, and costs roughly $30,000 [1]. After speaking with researchers who use these systems, we found reports of poor environmental control including large temperature gradients and CO_2 usage up to 20X less efficient than independent incubators. Temperature gradients are undesirable for cell culture, especially in microfluidic systems where small liquid volumes can evaporate unevenly.

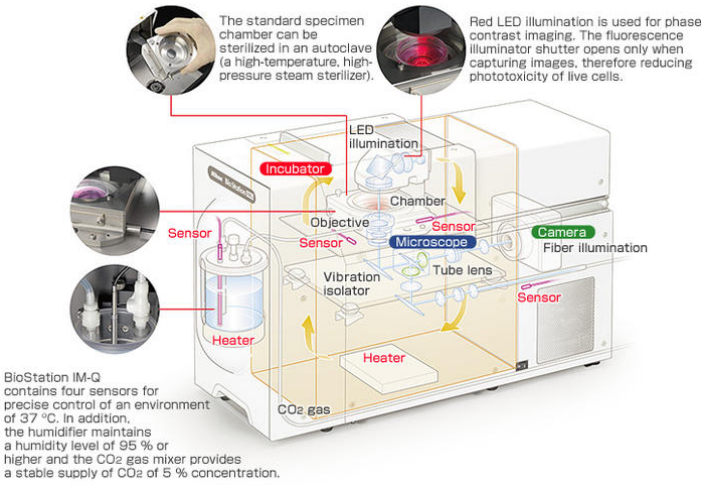

Figure 1: Nikon BioStation IM-Q Incubation system

Figure 2: Bioscience Tools Miniature Microscope Incubator

The second class of microscope incubators is comprised of small incubators which rest on the stage of an independent scope. This more affordable option, however, comes at the cost of reduced environmental control. An example product in this market class is the Miniature Microscope

Incubator by Bioscience Tools (figure 2) [2]. This system is a small, enclosed chamber that allows for inflow and outflow of air to maintain environmental parameters. Despite the relatively lower cost of just over $1000 for the entire system, the system has been reported allow large temperature gradients throughout the chamber. Despite the availability of both high and low-end cell culture incubation systems for use with live cell microscopy, products on both ends of the market currently fail to meet the needs of many researchers.

Problem Statement

The client desires an inexpensive on-stage incubation chamber for use in live cell imaging on an inverted microscope. The incubator should regulate temperature, Carbon Dioxide (CO_2), and humidity levels in the chamber with minimal gradients. The device should also be accessible for changing media and available for use with multiple culture dish types. In addition, the device should be able to be moved from the stage of one microscope to another, so as to not be limited to only one imaging system.

Background

Project Background

A full understanding of many aspects of cell biology requires observation of cell behavior over an extended period of time. Historically, such experiments were not possible due to lack of a physiological environment in which to observe cells.
It was not until the 1980's that Sally Temple, a researcher studying neural development in mice at the University of Florida, set out to observe neural progenitor cells for days at a time [3]. By building an incubator system around an old microscope, Temple was able to obtain time lapsed data over the course of a week.

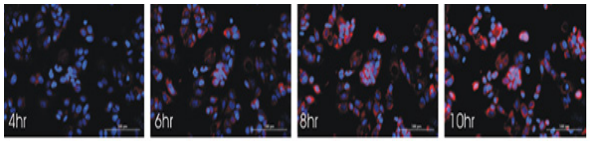

Figure 3: Time lapse fluorescent imaging at 1, 2, 3 and 4 hour increments using a scope integrated incubation system.

Today, the value in live imaging has been further realized. Through live imaging, researchers are able to perform experiments such as the one depicted in Figure 3, in which imaging takes place at small time increments, without exposing cells to the ambient environment at each sampling point [4].

Successful cell culture requires strict maintenance of three primary environmental parameters. These are temperature, humidity and CO_2 concentration.

Whether culturing mammalian cells or bacterial cells, optimal growth for the majority of cell culture will occur at 37□, the choice temperature for activity of most enzymes [5]. While methods of heat introduction vary throughout the market, stringent temperature control is one of the primary design considerations for all incubators. The ideal temperature control system will create a uniform temperature throughout the culture space.

Similarly, the pH of the *in vitro* environment will determine enzyme activity and thus cell viability [5]. Culture pH can be controlled via the concentrations of CO_2 gas, which diffuses into the culture media and maintains in equilibrium with bicarbonate, according to the following

equation: $CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows HCO_3^- + H^+$. The air CO_2 concentration can thus be controlled so as to maintain physiological pH (~ 7.4) in the culture [6].

The final primary environmental parameter controlled in incubators is relative humidity (RH). RH is used to reduce the vapor pressure of liquids in the culture. Reducing evaporation of culture media prevents change in salt concentrations (and thus pH). Maintaining a 95 - 100% humidity is vital to long term stability to media pH [7].

Unlike a system in which a microscope is placed inside of an incubator, an on-stage incubator presents structural limitations to the incubator. The system must comply with the smallest dimensions of any potential stage it might be placed on. In regards to optic dimensions, the design will have to allow adequate focal lengths for the desired magnifications as well as potential illumination sources used in imaging.

Design Specifications

Design specifications came from both the client and current market for independent incubator systems. After market review, our team presented the client with a number of design specifications representative of specifications for current culture incubation systems available. These were broken down into environmental control specifications and structural specifications. Using client feedback, the Project Design Specifications were documented, as seen in Appendix A. These specifications are summarized below.

Environmental Control Design Specifications

System must maintain temperature at 37□ ± 1□ , relative humidity within a 95-100% range, and CO_2 concentrations at 5% ± 0.5% [8] [9]. When in use, the culture will be opened for access to the user after which the system environment must recover to all set specifications within 10 minutes of a 30 second door opening [8]. Lastly, condensation from high humidity must not interfere with imaging.

Structural Design Specifications

The incubation chamber must be fixed to the stage of a Nikon Ti-U Inverted microscope, and used with an objective lens up to 20X magnification. Further, the system must accommodate both bright field and fluorescence microscopy.

Preliminary Designs

The microscope cell culture incubator, as stated previously, must incorporate elements to control and regulate the temperature, carbon dioxide, and humidity in the incubator's environment. The designs originally considered incorporated structural aspects to address this necessity, utilizing the MH-Z16 CO2 sensor, DHT-22 humidity and temperature sensor, heating pads, water reservoirs, carbon dioxide tank/valve, and fans. The first design, "Basic Box", essentially incorporates all of these elements into the single imaging chamber. The fan allows for continuous flow throughout the chamber to minimize gradients. The second design, "Dual Chambers", removes the heating elements, water reservoir, and carbon dioxide input from the sensors, and stores these elements into a separate box other than the imaging chamber, in an attempt to further reduce gradients and on-stage size requirements, while keeping the sensors in the imaging chamber. The final design, "The Moat", similarly to the "Basic Box" design incorporates all of the elements and sensors into the one imaging chamber, however lines the inner perimeter walls with the heating elements, and changes the water reservoir into a moat-like structure. This final design strives to promote better gradient control of the different elements by removing them indirectly from the imaging area.

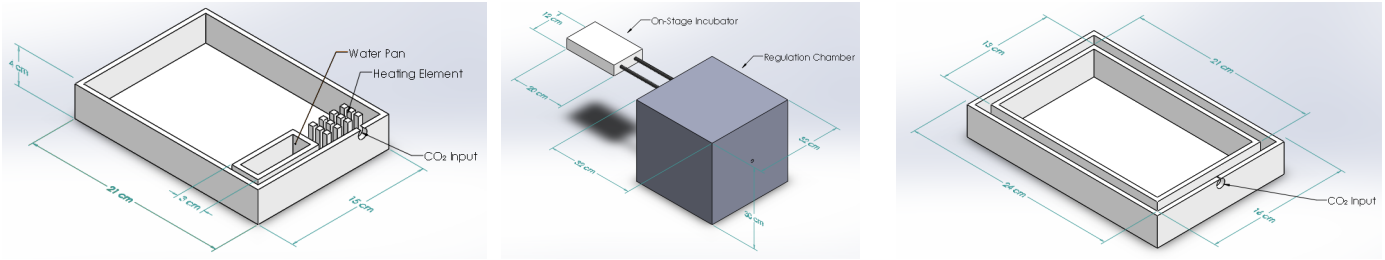

Figure 4: Preliminary designs. (Left) **"**Basic Box". (Middle) "Dual Chambers". (Right) "The Moat".

Preliminary Design Evaluation

The design matrix is based off of the client's specifications for the incubator. Heat regulation is weighed highest as heat must be evenly distributed throughout the incubator in order to prevent uneven evaporation, especially in microfluidic systems. CO2 and humidity regulation are weighed next highest, as CO2 monitoring is essential for proper cell growth and function. Further, humidity regulation plays an important role in cell growth, and prevention of evaporation in a warm environment. Ease of fabrication is the next most important criteria, due to the sheer number of sensors and environmental control components that are needed to be integrated into the design. Cost is after that, weighed only moderately as each design will have the same components included, and few variations in construction. Finally, accessibility and safety are weighed lowest, as accessibility is not of higher concern than environmental control of the incubator. Additionally safety, although important, is not of large concern in consideration

with each design, as any unsafe conditions (potential for burns, CO2 exposure), is already regulated by the environmental control categories, so these concerns are already evaluated.

The design the team ultimately made into the prototype was the basic box design, as it boasts advantages in environmental control regulation due to its smaller volume, as well as physical ease of fabrication. The design is appealing in its simplicity, and the basic box also offered us the ability to test each incubator component and collect data while constructing a prototype.

Fabrication/Development Process

Materials

The final prototype involved a combination of different electronic components and supporting hardware to create a functional incubator. A detailed list of materials can be found in Appendix C.

The feedback systems involved in regulating the chamber environment involved a number of electronic components. CO_2 was regulated through a JFSV00005 gas solenoid valve, which receives feedback through the MH-Z16 CO_2 sensor. Humidity was added with the Grove 101020090 water atomizer, and sensed via the DHT-22 temperature and humidity sensor. The DHT-22 also provided feedback to the heating system, which was regulated with heating pads. The heating pads and the solenoid valve were each controlled through a beefcake relay to account for their larger power supplies. A fan was included in the chamber to circulate air. All of these systems were integrated and controlled with an Arduino Mega microcontroller.

A number of casing components were used to contain the electronics involved in our prototype. The majority of the casing for the chamber itself was 3D-printed using ABS. The clear surfaces on the top and bottom were made of acrylic, which has low thermal conductivity and cost, without adversely affecting image quality. The lid for the chamber was attached via turn buttons. CO_2 was routed through tubing to the solenoid valve and then the CO_2 tank, and all the electronic components were housed in a separate box.

Methods

The prototype was created through an iterative process, in which smaller pieces were first developed before putting them together. Electronic components were each tested individually prior to sensors and regulatory hardware being paired and feedback loops being tested. Once feedback loops for each system were working, they were all tested in a single styrofoam box to ensure that they would work together. The casing and imaging platforms for the final prototype was simultaneously being developed, so once the systems were working in the larger model system, they could be integrated into the smaller final prototype. The casing was developed in the CAD software, Fusion 360, before being 3D printed with a Makerbot Replicator. The acrylic tops and bottoms were machined to size before being attached via acetone.

Final Prototype

Ultimately, the final prototype was assembled from a number of components including an acrylic base, plexiglas imaging regions, and two ABS 3D printed halves. The top region included four turn-buttons that sealed the enclosure, while still allowing access to the interior for changing media and refilling the atomizer water supply. The enclosure was then combined with sensors and feedback components, and the external circuit was housed in a separate box. Finally, soft feet to lift the enclosure off the stage, knobs to lift the lid off without having to touch the plexiglas, and a few aesthetic touched were added. To cover up exposed wiring, black braided sleeving was used.

In figure 5 (left), one half of the enclosure design is shown, all units are given in mm. The enclosure has grooves for the machined piece of acrylic on the bottom, as well as a .08inch piece of plexiglas. The acrylic insert also has a cutout which contours to two different cell culture plates. On the right, the spatial layout of all components before being affixed to the enclosure is shown. Additionally, the wiring and circuit is shown as exposed, whereas in the final prototype they are covered neatly.

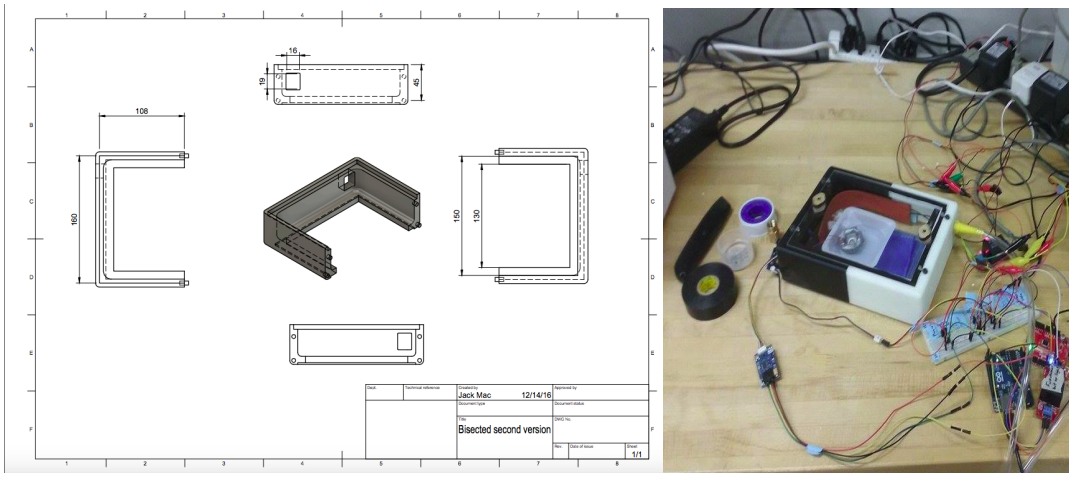

Figure 5: (Left) Layout and dimensions for the stage enclosure (black half) are shown. The other half is symmetric except for the hole for the CO2 probe. (Right) Picture of fully assembled final prototype without cosmetic changes added.

Testing

Electronics Function and Calibration

Electronic regulatory systems and feedback loops were first created individually using the sensor and corresponding response element. These systems were then tested individually in a single insulated styrofoam box which allowed for iterative testing to refine control of the system. Once properly calibrated to the degree of accuracy specified by our client, individual systems were combined for integrated testing inside the styrofoam box. After verification in this testing environment, the integrated components were transferred into our final prototype, where the same tests were validated. These tests were performed to determine whether or not this design met our client's requirements for controlling temperature, humidity and CO2.

Fully integrated design testing

More realistic testing was able to be accomplished within the final chamber prototype. After verifying that the electronics systems are still viable, short term tests were conducted along with a system disruption (opening and closing the chamber) providing data and allowing us to assess the system's ability to recover. Additionally, we observed the ability for the system to ramp up to the requirements specified by the client.

Optical Compatibility Tests

An important consideration in the design of the incubator was its ability to accurately capture cell images without decreasing incubation effectiveness. The materials considered for the design had optical properties similar to or the same as glass, with varying degrees of thermal conductivity [10]. In order to weigh the material choices, images were captured to determine if the refractive index or thermal conductivity values would be more limiting for the incubator's design. Figure 6 demonstrates the importance of considering refractive index for the design, as both the chosen material's ability to refract light as well as its thickness will contribute to changes in light incidence angles.

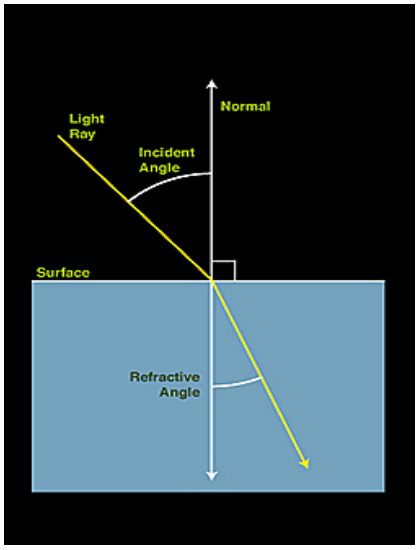

Figure 6: Effect of refractive index.

To test the compatibility of the materials listed in Table 1, the team took brightfield images of cells through 1.15 mm Polystyrene, 2.2 mm thick Glass, and 2.30 mm thick Plexiglass. Images were acquired for a control with just the cell culture flask, the flask on top of glass, the flask on plexiglas, and the flask on polystyrene, all at 20X magnification (Figure 9). Using MATLAB, (see code in Appendix D) percent of relative image focus was calculated and the materials were compared in Table 2, which is discussed in the Imaging Results section.

Material	Refractive Index (n)	Thermal Conductivity (k)
Polystyrene	1.55	0.03 W/mK
Glass	1.50	1.05 W/mK
Acrylic (Plexiglass)	1.517	0.20 W/mK

Table 1: Imaging material properties.

Results

Control System and Feedback Loop with Final Prototype

Figure 7 illustrates the results of our control loop testing for RH and humidity. The arrow and annotation indicates the point of interruption, where the chamber was accessed for 30 seconds. As expected, a significant drop in humidity can be noted, however temperature remained relatively stable. After the interruption, both temperature and humidity recovered well within the given specification. Another notable aspect of figure 7 is the fact that it takes a significant amount of time for RH levels to reach the point that we desire. This means that during cell trials, we will need to ensure that the enclosure has become humid enough before introducing our cells.

Temperature remained fairly constant throughout the experiment, and the steady state region had an average temperature of 37.07 □ with a standard deviation of .56□. This falls is within our specification of 37□ ± 1□. However, humidity behaves more erratically and before it has fully ramped up to the desired humidity level the incubator is opened.

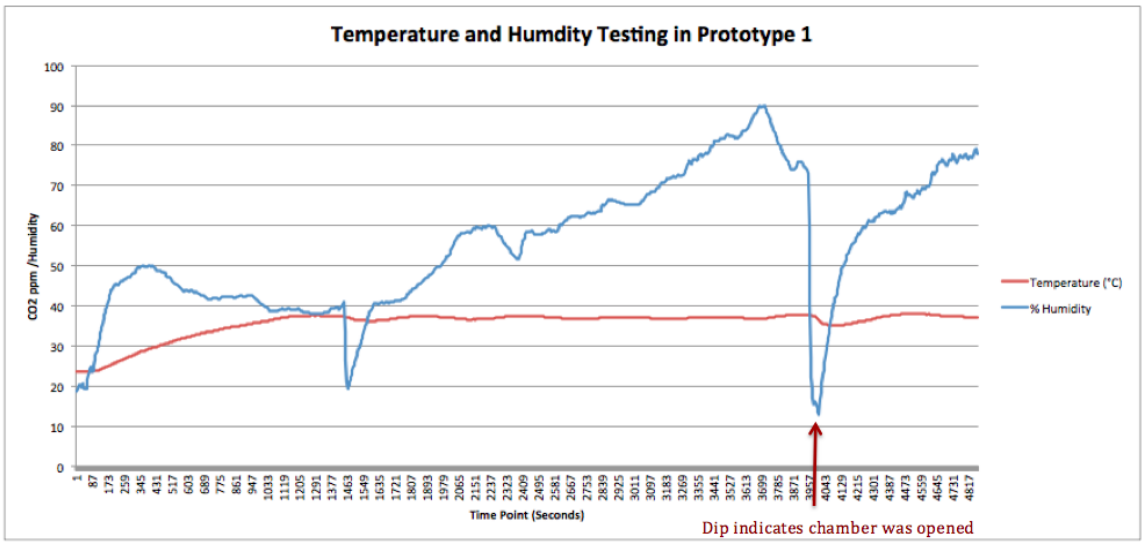

Figure 7: Graphs of RH and temperature over time. At the arrow, the enclosure is opened to determine the impact it has on each control.

Similarly, CO2 levels over time in the final enclosure are depicted in Figure 8. Over time, the CO2 level remains fairly constant until the interruption occurs, and when the incubator is closed again it returns to the set point without issue. While our probe did not have the correct range to detect CO2 at the level specified by our client (5%), we were able to use the same model probe with a different detection range (0-10,000ppm) to keep CO2 levels constant. As seen in the graph, once CO2 reaches the level that we intended, it stays very consistent until the system is opened (noted by the sharp dip). Once the incubator was closed again, CO2 very effectively returns to 1% within a time period of less than ten minutes. This is encouraging, and validates our ability to control CO2 in the incubator as well as be able to return to our control. Analysis of the data shows that we had an average CO2 level of 6518.9 ppm, with a standard

deviation of 55.4ppm. This leads to a percent error in the steady state region of ~0.85%. The parameter given by our client was 5% ± 0.5% atmospheric CO_2, which is a percent error of 10%. Our obtained value is significantly lower than the allowable tolerance, which reaffirms the consistency of our feedback loop.

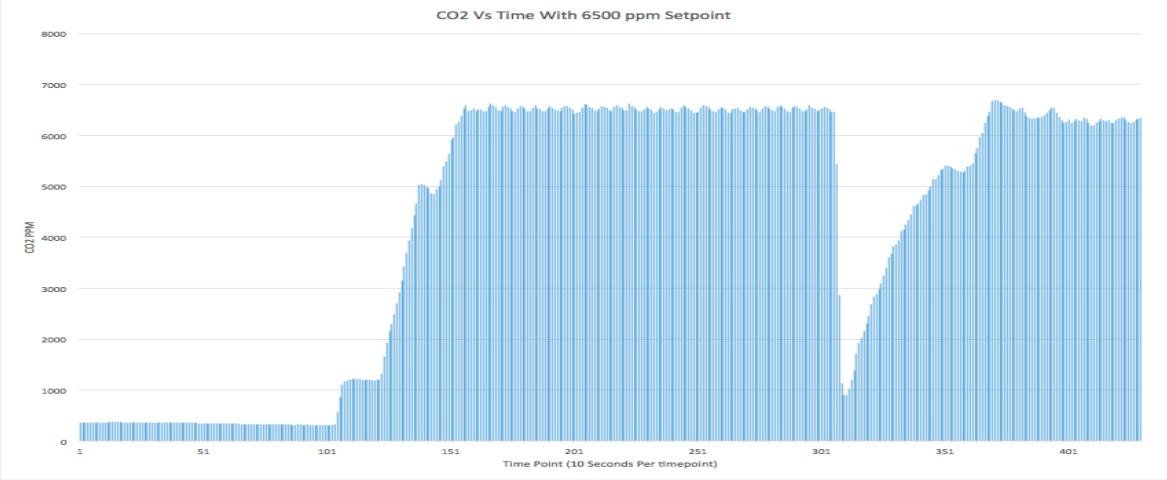

Figure 8: Graph of CO2 over time. At the drop, the enclosure was opened to determine the impact it has on maintaining CO2 levels internally. Over the next ten minutes, CO2 returns to the set value.

Imaging Results:

Results from the MATLAB analysis of relative image focus are shown below in Table 2, as well as the images taken for each case (Figure 9). The MATLAB function, f_measure, compared local contrast values between pixels for each image in grayscale, and then computed an overall image focus calculation for the image. Further information about the code used for these tests can be found in Appendix D. The four test conditions used were chosen to evaluate three standard imaging materials to a control condition: viewing cells in a tissue culture polystyrene (TCPS) culture flask. Although none of the materials were able to create images with as high of relative image focus as the control, it was clear that both the acrylic and glass conditions outperformed polystyrene in image quality. As mentioned in Optical Compatibility Tests, the team had to also consider the material's impact on heat maintenance in the system. Due to acrylic's much lower thermal conductivity (k = 0.20 W/mK) and improved shatter resistance, the team chose acrylic over glass as the final imaging material.

Material Type and Thickness	% of Relative Image Focus
Control, t = 0 (A)	37.44%
Glass, t = 2.2 mm (B)	23.27%
Plexiglass, t = 2.3 mm (D)	22.14%
Polystyrene, t = 1.15 mm (C)	16.56%

Table 2: Percent Relative Image Focus of different materials

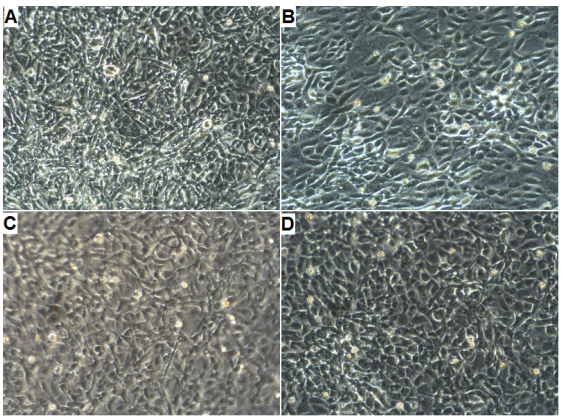

Figure 9: Images taken of cells in a culture flask through various materials. (A) No material control. (B) Glass. (C) Polystyrene. (D) Plexiglas.

Discussion

This first design iteration was able to regulate all three major environmental parameters identified in the design specifications: temperature, relative humidity, and CO_2. Both temperature and relative humidity were maintained within the limits of the design specifications. While testing proved the control system accuracy and stability over time, the CO_2 set point, 0.65%, was significantly below the desired specification of 5%. Although we could have injected enough CO_2 to reach this concentration in the chamber, the sensor that was purchased was unable to detect any amount of CO_2 greater than 1%. Increasing this set point will be a trivial test once a new sensor with a higher concentration limit has be obtained. Additional focus will be placed on accurate and physiologically relevant maintenance of CO_2 in the coming semester, which will be discussed further in future work.

In addition to the system's ability to reach and maintain desired environmental set points, the results also indicate that the temperature and CO_2 systems were able to recover to these conditions within the 10 minute specification. There was a large initial delay before humidity reached its desired range, and after a disturbance, the humidity lagged behind both temperature and CO_2 in its recovery. If the system was turned on prior to incubation, the slow humidity ramp-up would not be an issue. However, the large delay after chamber opening could compromise the health of the cells in the incubator. Due to the inability to monitor CO_2 above 1%, delay in humidity recovery, and the short test durations, the device in its current state could not be successfully marketed as a competing product to other live cell culture incubators. However, the main accomplishment for the semester was proof of concept both in the chamber's design and its ability to maintain and recover heat, humidity, and CO_2 systems based off of sensor feedback.

From the results of the incubator systems testing, there have been a few key areas identified for modification in order to resolve the previously listed limitations. First, a new CO_2 sensor should be used that can measure up to and above the desired 5%. Next, a more robust humidity recovery mechanism can be investigated, such as a larger dish of water from which both evaporation and atomization can occur. Finally, a longer testing time frame must be used to ensure long-term system stability and verify that the sensors monitoring the system are not significantly drifting from calibrated values. Further modifications to the design and electronics control system will be described in future work.

Throughout testing, a large potential for error was introduced through use of system-dependent sensors to monitor environmental conditions. Although the sensors that were purchased have been calibrated by their respective manufacturers, they could still be sensing inaccurately or drift from calibrated values over time. One problem noticed early in the semester was the first temperature/humidity sensor (DHT22) purchased could only reach measurements up to 94.20% humidity. While this problem was eventually resolved after purchasing a new sensor, the investigation into the issue revealed that the sensor itself detected condensation on its surface. If the system experienced a fast drop in temperature during use, the resulting condensation on the humidity sensor would artificially increase the sensor's humidity readings, stopping controllable water vaporization. While there was no indication that this would occur based on the results, it raises concerns about the use of only a single, system-dependent sensor to control the incubator environment. Outside of sensor limitations, additional sources of error could include only measuring temperature, humidity, and CO_2 values at one location throughout testing, as well as potential air leaks in the incubator due to fabrication limitations. One key consideration the client listed was the elimination of temperature and humidity gradients throughout the incubator to reduce evaporation from microfluidic devices. More testing must be performed to measure temperature gradients during incubator operation and after opening before this can be addressed further.

Conclusions & Future Work

The goal for the semester was to design and build an incubator that could easily fit on the stage of a microscope for live cell imaging. Although this is not a novel idea, products available on the market are either very expensive, or not reliable enough to be used for studies lasting up to the desired two weeks. The final design created this semester will help to bridge this gap in the market. The "yin-yang" incubator is a single chamber system that can easily fit on the stage of an inverted Nikon microscope, regulates temperature, humidity, and CO_2, and allows for clear images to be captured at a single spot in the cell culture dish without noticeable condensation on the imaging surfaces. In addition to these attributes, the incubation system was constructed under the budgeted $200 (See Appendix C for costs). After testing the device, it is clear that there is potential for the single chamber system to perform as well as standard cell culture incubators on the market, without the cost. However, various components in the system must be modified in order for the system to function as well as available culture systems. Improved monitoring of CO_2, faster humidity recovery, and larger test durations will improve the system's performance and reliability.

Aside from changes to the system's environmental controls, there are other changes that could be made to the system to improve its usability. When performing live cell imaging with the final design, it was very difficult to transport the chamber from bench top to microscope stage because of the attached circuitry. To improve ease of use, the team will streamline the current electronics system by integrating the controls into a printed circuit board. The various sensors and environmental control elements inside the incubation chamber also made sterilization difficult for use with cells. Ideally, the chamber should be easily sterilized using ethanol or even autoclaving. To facilitate sterilization, the team will work to isolate the sensors and other electronics from the cell culture area. Two paths for this could either be creation of a removable cartridge loaded with sensors and electronics, or a separate chamber from which heat, humidity, and CO_2 can be introduced and monitored.

Finally, more robust testing must be performed to demonstrate the system's ability to eliminate temperature gradients and maintain ideal cell culture and imaging capabilities for at least two weeks. In the next semester, a device will be used to visually display a heat map of the chamber during use. This will qualitatively determine regions where heat must be better distributed in the system, so additional fans or heating elements can be added. After the system is modified, it will be tested with live cells for at least two weeks, with imaging and cell culture protocols performed intermittently throughout this period. Testing can also be conducted to demonstrate that the system can be easily moved and used with multiple microscopes. After these modifications are made and additional testing is performed, the incubator will hopefully be able to perform to the standards of other cell culture devices on the market, with a much lower price tag.

Many milestones were achieved throughout the semester towards the creation of an affordable, versatile incubator for live cell microscopy. The final design was able to sense and alter chamber temperature, humidity, and CO_2 based on environmental changes, while the imaging platform allowed for successful image capture despite changes in focal length. Although modifications to the system are required to meet and exceed the initial design specifications, these can be accomplished within the scope of the next semester. Upon completion of this project, a new device will be available for affordable and reliable live cell imaging. This device will bridge the gap in the incubation market, allowing researchers and students access to technology otherwise unavailable.

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Appendix

Appendix A: Product Design Specifications

Function: The device should enable the continuous culture of live cells for up to two weeks on an inverted microscope, without impeding imaging capabilities. The cell culture environment must imitate that of an incubator with precise control and readout of temperature, CO_2 mixture, and humidity all within a sterile environment.

Client Requirements:

- Temperature control and readout
- Humidity control and readout
- CO_2 concentration control and readout
- Incubation container must not impede ability to image
- Accessible for changing cell culture plates and changing media
- Sterilizable with a standard 70% ethanol solution
- Fit securely on an inverted microscope as to ensure imaging of a consistent location

Design Requirements:

1. **Physical and Operational Characteristics**
    a. ***Cell Culture Related Performance Requirements:*** The device should maintain incubator-like conditions for 2 weeks. It must maintain the temperature at 37□ ± 1□, and reestablish temperature after less than 6 seconds following a 30 second door opening. It must maintain 95-100% humidity within culture chamber. Finally, it should maintain 5% ±0.5% CO_2 concentration and reestablish concentration after less than 6 seconds following a 30 second door opening.
    b. ***Incubator Housing Related Performance Requirements:*** Incubator housing and any potential condensation must not disrupt optics during imaging. The housing must not limit ability to navigate the full field of the cell culture plate, and not substantially change the distance between the cell culture plate and the objective. Housing materials must be compatible with culture media and be sterilizable with 70% ethanol solution. The system should also have adequate insulation to prevent internal temperature fluctuations due to external temperature changes.
    c. ***Safety:*** Culture environment must be compliant to BioSafety Level 1 standards. All electrical components within the culture environment must be sterile and waterproofed, and all circuitry must be rated to the supplied power and current used.
    d. ***Accuracy and Reliability:*** The precision of the system components is outlined in the performance requirements listed above. For each of the four environmental parameters we will be controlling (temperature, humidity, CO_2 percentage and air

sterility), the combined error of sensor measurement/readout and parameter control must be within the tolerance. The precision measurements taken during system use are as follows:

i. Humidity: 95-100% humidity
ii. CO_2 concentration: 5% ± .5% of readout value
iii. Temperature: 37□ ± 1□ of readout value

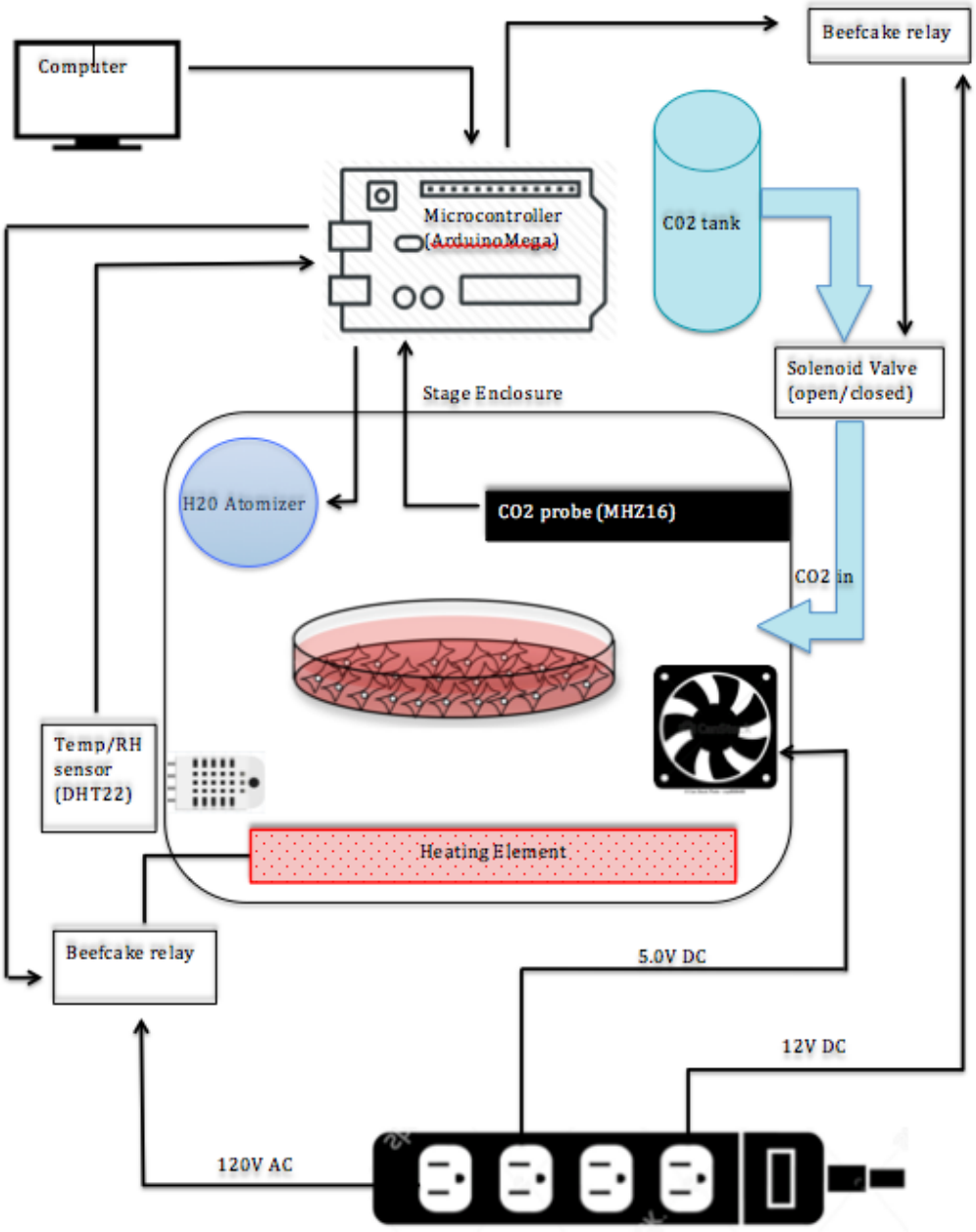
e. ***Life in Service:*** The incubation chamber should maintain the specified environmental conditions to promote cell life for up to two weeks. The internal portions of the design under these environmental conditions must function without recalibration or repair during this time period.
f. ***Operating Environment:*** The internal portion of the incubation chamber must function in conditions of 95% relative humidity or more, temperatures of 37□ and CO_2 levels of 5% during incubator operation. If the system is not in operation, the incubator will be exposed to normal environmental conditions: room temperature, environmental humidity, and low CO_2 concentration. There will be limited dirt exposure inside the incubation chamber, as live cells will be stored in it. Users will be opening and closing the incubation chamber, so the system will also have to adapt to sudden drops in temperature, relative humidity, and CO_2 percentage. It must be possible for the user to change media for cells inside the incubation chamber without changing the location that is being imaged.
g. ***Ergonomics:*** The user will have limited interaction with the incubator itself, except to move cell culture dishes in and out of the chamber. The door to the chamber should be easy to open, and allow for enough clearance to fit a cell culture plate, flask, or petri dish inside the incubator.
h. ***Size:*** The interior of the incubation chamber should be at minimum 15.4 cm x 9.4 cm x 2.5 cm tall. The incubation chamber should fit securely on a stage with dimensions as small as 16.0 cm x 25.0 cm, with a clearance of 5.3 cm tall for the light source.
i. ***Weight:*** Each component of the final product should be no more than 12 kg, such that it is easy to transport between experiments without too much difficulty.
j. ***Materials:*** Materials used for the incubation chamber should not have cytotoxic effects on cells inside their culture dishes, and should be sterilizable with ethanol. The materials should be resistant to corrosion from the high humidity levels. We must use glass for the bottom surface, and the top surface should not defract light from the light source significantly.
k. ***Aesthetics, Appearance, and Finish:*** The surfaces through which imaging will occur should be transparent, and not result in any aberrations or otherwise compromise the quality of imaging. There should also be a mechanism to protect the experiments from light pollution.

2. Production Characteristics

a. ***Quantity:*** The client needs a total of one microscope cell culture incubator.
b. ***Target Product Cost:*** The target product cost is to be $200, with an understanding that the product would enter the market for around $500.

3. **Miscellaneous**
    a. ***Standards and Specifications:*** There are no standards and specifications to our knowledge that must be addressed within the design.
    b. ***Customer:*** Any customer preferences are already addressed above and have been taken into consideration.
    c. ***Patient-related concerns:*** The product will not have any contact with patients, so patient-related concerns are not applicable.
    d. ***Competition:*** There are a variety of systems that have been fabricated for similar purposes, but to the knowledge of the team the device we intend to create would be unique in cost, ease of use, and the ability to be used with a number of microscopes. Stage incubators on the market, such as the Pecon Incubation System 2000 fits all functional requirements of the client but is specifically tailored to fit the Olympus IX71/81 microscope. Ideally, we will be able to translocate our final prototype from one imaging system to another and it will be relatively universal. The majority of available systems also enclose the whole microscope, which limits the system's versatility.

Appendix B: System Control Diagram



Appendix C: Materials List and Expenses

* Note that all components without associated price were provided through the client for free. The assigned project budget relates to spending on top of these components provided.

Materials List

Category	Material:	Price:
Sensors	MH-Z16 CO_2 Sensor	$67.95
	DHT-22 Temperature/Humidity Sensor	$9.95
Regulatory Electronics	JFSV00005 (¼") Gas Solenoid Valve	$11.99
	Grove 101020090 Water Atomizer	$9.90
	Heating Elements	-
Other Electronics	Arduino Mega Microcontroller	-
	Beefcake Relay (2)	-
	Fan	-
	Wiring	-
	Breadboard	-
	Wall Power Supplies	-
Casing Materials	Acrylic Plastic Sheet - 12x12x0.08	$5.14
	ABS Casing	-
	Handles (for lid	-
	Turn Buttons (for lid)	-
	Electronics Housing	-
	Cable Wrap	-
CO_2 Hardware	CO_2 Tank	$18.56*
	CO_2 Tank Valve/Gauge	-
	Tubing	-
	Tubing Adapters	-
Total:		**$123.49**

Client Purchases

Item:	Cost (including shipping):
MH-Z16 CO_2 Sensor	$67.95
JFSV00005 (¼") Solenoid Valve (2)	$33.98
DHT-22 Temperature/Humidity Sensor (3)	$43.94
Grove 101020090 Water Atomizer (2)	$19.80
CO_2 Tank	$18.56*
Acrylic Plastic Sheet - 12x12x0.08	$5.14
TIP120 Power Transistors (2)	$5.00
Total:	**$194.37**

*CO_2 tank rental costs an additional $4.62 per month.

Appendix D: Microcontroller and Matlab Code

Matlab Image Analysis Code

```
%Image Analysis Code using fmeasure library in Matlab
addpath('/Users/jennywestlund/Downloads/fmeasure/fmeasure/fmeasure.m');

%Read in images from files
ControlI = imread('20XControl-1.tif');
GlassI = imread('20XGlass(use)2.tif');
PlexiglassI = imread('20XPlexiglass2.tif');
PolystyreneI = imread('20XPolystyrene2.tif');

%Convert images to grayscale
GrayControl = rgb2gray(ControlI);
GrayGlass = rgb2gray(GlassI);
GrayPlexiglass = rgb2gray(PlexiglassI);
GrayPolystyrene = rgb2gray(PolystyreneI);

% Analyze Image focus based on Brenner's focus measure operator, outputs
% relative degree of focus percent of the image
FM1 = fmeasure(GrayControl, 'CONT')
FM2 = fmeasure(GrayGlass, 'CONT')
FM3 = fmeasure(GrayPlexiglass, 'CONT')
FM4 = fmeasure(GrayPolystyrene, 'CONT')
```

Arduino RH and Temperature Code

```
#include "DHT.h"

//DHT22 PIN-OUT GUIDE
// Connect pin 1 (on the left) of the sensor to +5V
  // NOTE: If using a board with 3.3V logic like an Arduino Due connect pin 1
  // to 3.3V instead of 5V!
// Connect pin 2 of the sensor to whatever your DHTPIN is
// Connect pin 4 (on the right) of the sensor to GROUND
// Connect a 10K resistor from pin 2 (data) to pin 1 (power) of the sensor

#define DHTPIN 2     // what digital pin the DHT22 is connected to
```

```
#define DHTTYPE DHT22
const int heatPin = 12; //Assign pin 12 to the relay for heat control
const int ledH2O = 13; //Assign pin 13 to the atomizer

// Initialize DHT sensor.
DHT dht(DHTPIN, DHTTYPE);

void setup() {
  pinMode(heatPin, OUTPUT);
  pinMode(ledH2O, OUTPUT);

  pinMode(51, OUTPUT);
  pinMode(52, OUTPUT);
  pinMode(53, OUTPUT);

  Serial.begin(9600);
  Serial.println("DHTxx test!");

  dht.begin();
}

void loop() {
  // Reading temperature or humidity takes about 250 milliseconds!
  // Sensor readings may also be up to 2 seconds 'old' (its a very slow sensor)

// Power supply for Heating Element relay, DHT22, and atomizer
 digitalWrite(51, HIGH);
 digitalWrite(52, HIGH);
 digitalWrite(53, HIGH);

  float h = dht.readHumidity();

  float hScaled = h * 1.06157113;

  // Read temperature as Celsius (the default)
  float t = dht.readTemperature();
  // Read temperature as Fahrenheit (isFahrenheit = true)
  float f = dht.readTemperature(true);

  //Heating the system: power for 500ms, rest for 2100ms
  if (t < 32.00) {
    if (millis() % 2600 < 500) {
      digitalWrite(heatPin, HIGH);
    } else {
```

```
    digitalWrite(heatPin, LOW);
   }
  } else {
   digitalWrite(heatPin, LOW);
  }

  //Feedback loop for RH
  if (h <= 70.00) {
     digitalWrite(ledH2O, HIGH);
  }else {
   digitalWrite(ledH2O, LOW);
  }

  // Check if any reads failed and exit early (to try again).
  if (isnan(h) || isnan(t) || isnan(f)) {
   Serial.println("Failed to read from DHT sensor!");
   return;
  }

   Serial.print("Humidity: ");
   Serial.print(hScaled);
   Serial.print(" %\t");
   Serial.print("Temperature: ");
   Serial.print(t);
   Serial.print(" *C, ");
   Serial.print(f);
   Serial.print(" *F\n");
}
```

Arduino CO2 Sensor Read In and Control

```
#include <NDIRZ16.h>
#include <SoftwareSerial.h>
const int GoalCO2 = 7500;
double CurrCO2=0;
int CO2Pin = 7;

//Arduino UNO Pin D2 (Software Serial Rx) <===> Adaptor's Green  Wire (Tx)
//Arduino UNO Pin D3 (Software Serial Tx) <===> Adaptor's Yellow Wire (Rx)
SoftwareSerial mySerial(2,3);
NDIRZ16 mySensor = NDIRZ16(&mySerial);

void setup()
```

```
{
   Serial.begin(115200);
   mySerial.begin(9600);
   Serial.println("Wait 10 seconds for the sensor to starup");
   delay(10000);
   pinMode(CO2Pin,OUTPUT);
}

void loop() {
   CurrCO2 = mySensor.measure();
   if (mySensor.measure()) {
      CurrCO2 = mySensor.ppm;
       Serial.println(CurrCO2);
       if (CurrCO2 < 7500){
         digitalWrite(CO2Pin, HIGH);
         delay(10);
         digitalWrite(CO2Pin,LOW);
       }
   }

   delay(5000);
}
```