Affordable cell-culture incubator for real-time imaging

Jack McGinnity, Steve Gock, Trevor Zarecki, Jennifer Westlund & Peter Hartig

Biomedical Engineering Department, University of Wisconsin-Madison

1550 Engineering Dr, Madison WI, 53706

Author Note: All funding for this project was provided through the project client, Dr. John Puccinelli.

Keywords: Incubator, cell-culture, environmental control, real-time imaging

Summary

Long-term live cell imaging experiments are difficult to perform on normal lab microscopes without imaging capabilities. Current commercial microscope-based incubators exist to answer this problem but have significant limitations. They are often expensive, limited to one microscope's imaging capabilities and cell plate size of just one microscope, and often ineffective at evenly controlling the environment. This project aims to create an affordable incubation chamber compatible with inverted microscopes that is capable of evenly maintaining desired temperature, CO_2, and humidity over a period of up to 2 weeks. Prototype testing has demonstrated adequate regulation of these three systems, through automated feedback control capable of maintaining the system near a physiological set point. Microscope compatibility testing has verified that imaging capability was maintained during cell culture. Further development of the design will ensure that it consistently performs efficiently according to all specifications, and ultimately will help bridge the gap in the market between high-cost, functional incubation systems and cheaper, less effective designs.

Introduction

Observing cells in an environment that exactly mimics human *in vivo* conditions is the ultimate goal for *in vitro* cell culture observation. In line with this goal, minimizing the effect of removal from the incubation chamber has historically been an important consideration for long term *in vitro* studies. (Rodriguez, 2005).

While significant progress towards this goal has been made through technologies such as enclosed microscopes, a gap in this technology persists in the area of real-time culture observation. Despite the ability to cinematically observe cell cultures over many generations, real-time culture imaging remains difficult for many of today's researchers. Two primary factors

have prevented the adoption of this technique: cost and cell culture quality. The cost for an industry quality, integrated microscope incubator system begins on the order of $30,000 and can quickly exceed $100,000. For many researchers, real-time imaging would enhance their experimental data but is not vital to their experiments, and thus this cost has limited the market for such a system. While more affordable alternatives such as the Warner Instruments DH-40iL ($1214) (Warner, 2017), exist that allow for users to place the incubation system directly onto a microscope, the current available systems do not provide the stringent and consistent environment required for publication quality data collection.

Aware of the numerous benefits real-time imaging has to offer, the client for this project, Dr. John Puccinelli, requests the design of an on-stage cell culture incubator with environmental regulation meeting the standards of today's incubator market leaders. This system must also fall into a price range that will make it accessible to researchers interested in receiving the benefits of this technique without the significant investment.

Materials and methods

Casing Assembly

The outer casing for the incubator was designed in Fusion 360 and 3D-printed in ABS with a Makerbot Replicator 2X, as shown in **Figure 1**. Two separate halves were printed and adhered to each other using acetone. Ports were created in the ABS to allow for CO_2 gas injection, the CO_2 sensor, and electronics wiring. An ABS printed lid with an acrylic insert was used for the top imaging surface to retain heat in the system and allow for users to view the cells. A 10.28mm x 12.85mm x 0.24mm piece of glass was used for the bottom surface. Additionally, a cutout in the bottom of the incubator casing over the glass surface secured in place a standard

cell culture plate and 10 cm petri dish such that they remained in place within the incubation chamber.

Electronic Circuit

Control of the environment was maintained using the hardware as shown in **Figure 2**, and circuitry configured as illustrated in **Figure 3**. Power was supplied to all circuit elements from the VGD-30-D512 multiple output (12V and 5V) AC DC converter. A printed circuit board including relays, supporting circuitry, and the ATmega328 microcontroller was used to control circuit elements within the feedback system. Pin headers and a ribbon wire attached the exterior electronics box to the on-stage incubation chamber. The DHT22 temperature/RH sensor was used to sense heat and relative humidity (RH). An immersion heating element in conjunction with a relay was used to control the temperature of the system and introduce water vapor. CO_2 was measured with the MH-Z16 CO_2 sensor; a JFSV00005 gas solenoid valve, in conjunction with another relay, was used to control gas flow from the CO_2 tank into the incubator. Finally, a small fan provided air circulation within the system.

Software

A feedback control loop was used to adjust the CO_2 injection rate and temperature appropriately, depending on the input from sensors. Code was designed to cause larger additions and thus larger increases in CO_2 or temperature following openings of the chamber. This system also allowed for much smaller adjustments to be made during regular operation.

Cell Culture

Cells used for incubation testing were bovine aortic endothelial cells (BAECs). The cells were kept in a Sanyo incubator at 37°C, 5% CO_2, and approximately 90% relative humidity prior to culture in the final incubator prototype. Cells were grown in cell media composed of DMEM

(Sigma) with 10% Fetal Bovine Serum (FBS), and 1% penicillin-streptomycin (pen-strep). Protocols for cell re-feeding and passaging are attached in the appendix. Cells were used up to the 10^{th} passage, with a recommended passage length of 14 for BAECs.

Scratch Assay

All procedures follow the protocol described in *Liang et al.* (Liang, 2007). In this process, cells were cultured to confluence (80-100% cell coverage) prior to applying the wounding scratch across the Tissue Culture Polystyrene (TCPS) dish. Following wounding, media was aspirated and replaced to remove non-adherent cells. Cells were then placed into one of the three different conditions. The first condition, the positive control, was placed in the Sanyo incubator, the experimental condition was cultured in the final prototype incubator, and the negative control condition allowed cells to sit at ambient temperature, humidity, and CO_2.

Results

Environmental control

To test environmental control in the preliminary prototype, temperature, humidity, and CO_2 measurements were taken over the course of about 6 hours. **Figure 4** displays the measurement curves for temperature, CO_2 concentration and humidity. In addition to steady state measurements, the data includes four instances of a 30 second chamber opening after which parameters were monitored for recovery to steady state levels.

Imaging

One significant 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 must have optical properties similar to glass (positive control), good mechanical stability in the incubator environment, and a low thermal conductivity (Interactagram, 2009). In order to

evaluate 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. The refractive index for the design was an important consideration, as both the chosen material's ability to refract light as well as its thickness contributes to changes in light incidence angles.

Image tests for the previous prototype were performed by taking bright field images of cells through a TCPS control, 1.15 mm polystyrene, 2.2 mm glass, and 2.3 mm acrylic. The images were then analyzed in MATLAB to determine an overall focus value between the tested conditions. Results from the MATLAB analysis of relative image focus are displayed alongside the images taken for each case in **Figure 5**. The MATLAB function, f_measure, compared local contrast values between pixels for each image in gray scale, and then computed an overall image focus calculation for the image. 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.

Glass was ultimately used as the imaging surface in the final prototype. Although acrylic performed similarly in both relative image focus and thermal conductivity to glass, it was found that the material scratched easily and was prone to ethanol degradation. Tests for image quality on glass showed a percent focus value of 31.8641%, while the percent focus for the image taken through just the standard TCPS plate was 31.0734%. This test demonstrated that clear images could be captured through a glass surface at 20X of magnification, which meets the design requirements and validates the use of glass in the final design.

Scratch Assay

While experimental design of the scratch assay does not allow for reliable quantification of results, qualitative results can be observed. Images of cell wound healing over a 4 hour time

period are displayed in **Figure 6.** As expected, cells held at ambient conditions, post wounding, displayed very little wound repair (ingrowth migration). Further, many of the cells for this condition become detached from the cell culture dish near the end of the 4-hour time frame. In contrast, cells incubated in both the standard Sanyo incubator and the prototype described here illustrated wound repair. However, cells in the Sanyo incubator displayed more substantial repair. One possible reason for this difference was due to pH buffering issues in the experimental incubator. Despite CO_2 values being maintained at 5 ± 1% for almost the entire test duration, the color of the DMEM-based media began to turn from a light pink to a darker, yellow/brown, as demonstrated in **Figure 7**. This color change indicates an increasingly acidic pH, which is due to exceeding the necessary environmental CO_2 levels. The set point for CO_2 was adjusted to 4% after 3 hours of testing and cell media was replaced, however the media still demonstrated acidifying. This may indicate need to experiment with correct CO_2 refinement in the future, or to recalibrate the sensor used for this purpose. Despite issues with CO_2 buffering, the cells remained viable throughout the course of the testing, as indicated by their ability to migrate into the scratch and retained adherence to the TCPS.

Discussion

Environmental Control

Results from this test indicate that both temperature and humidity control meet design specifications for successful cell culture. Further, the recovery times for heat met the 10-minute recovery specification and the humidity recovered from the opening in only 1 minute (again meeting specification). The primary difficulty encountered during culture was stability of CO_2 concentration. While the MHZ –16 was specified to measure with a 200 ppm accuracy and the data in **Figure 4** shows relatively steady state CO_2 measurement of 50,000 ppm (5%), during cell

culture media was observed to yellow. This indicates a rise above the set pH of 7.4 and was the likely factor behind decreases wound healing observed in the test sample. The results of this testing suggest that the MHZ-16 were not stable in measurement. As a result, the feedback loop controlled by its output allowed for fluctuations in concentration.

A second difficulty encountered in CO_2 control was the lack of control over pressure behind the CO_2 valve. The pressure gauge was manually controlled and as a result, made it difficult to achieve consistent feedback control when opening the solenoid valve to increase CO_2 concentration.

Imaging & Materials

While the work done in the last semester indicated that an acrylic material, or Plexiglas, would be an ideal image surface, further research has indicated two issues with this choice: sterilization and scratching. The desired sterilization method for the incubator is use of 70% ethyl alcohol, which is commonly found in sterile laboratories and can be applied quickly and easily. However, various sources have indicated that acrylic materials degrade with prolonged exposure to alcohols (RTP Company). Any type of material degradation, including scratches, will alter image quality over time. In order to avoid reduced image quality after repeat sterilizations, the next generation prototype was constructed with glass as the imaging surface instead of acrylic. Despite potential heat loss from glass's higher thermal conductivity, the incubator was able to maintain consistent temperature throughout the testing duration. From this modification, the system will be able to withstand many sterilizations without reduced image quality.

Conclusion & Future Work

Many milestones were achieved towards the creation of an affordable, versatile incubator for live cell microscopy. The design that has been created is able to sense and alter chamber temperature, humidity, and CO_2 to relevant physiological conditions based on environmental changes, while the imaging platform allows for successful image capture despite changes in focal length. However, longer-term environmental tests and adjustments to the CO_2 buffering must be performed prior to application in research. Upon refinement 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, providing researchers and students access to technology otherwise unavailable.

Acknowledgements

Special thanks to Prof. Mitch Tyler, Kevin Eliceri and Dr. John Puccinelli for their advice and resources in the development of this project.

References

Interactagram. (2009, 5 28). *Refraction - Snell's Law*. Retrieved from Interactagram: http://interactagram.com/physics/optics/refraction/

Liang. (2007). In Vitro Scratch Assay: A convenient and inexpensive method for analysis of cell migration in vitro. *Nature Protocols* , 329-333.

Rodriguez, L. G. (2005). Wound-healing assay. *Cell Migration: Developmental Methods and Protocols* , 23-29.

RTP Company. (n.d.). *Chemical & Environmental Resistance of Thermoplastics*. Retrieved from RTP Company: https://www.rtpcompany.com/technical-info/chemical-resistance/

Warner, I. (2017). *Micro-Incubation System (DH-40iL)*. Retrieved from Warneronline.com.

Figures

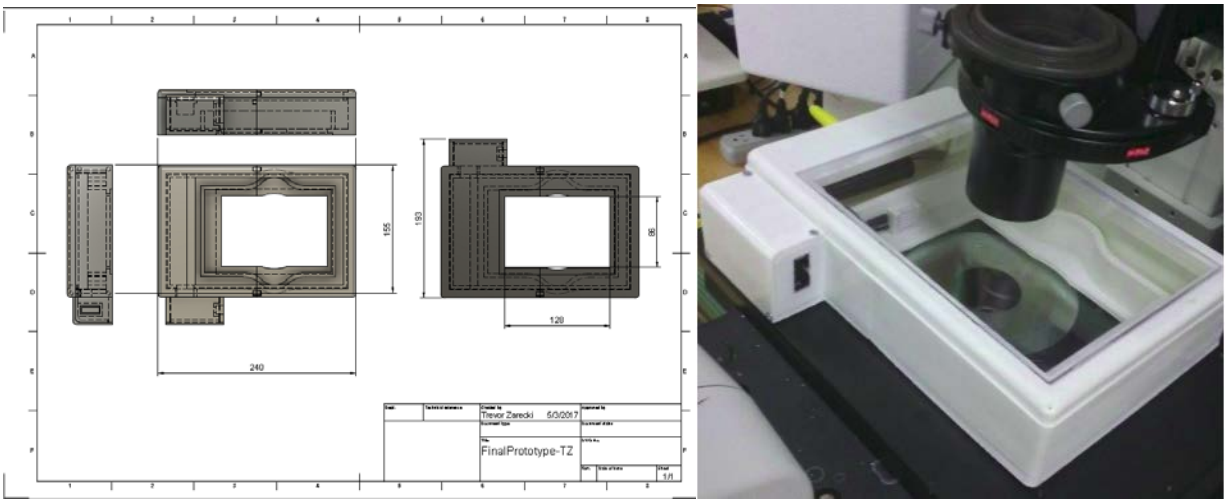

Figure 1. Incubation chamber for final prototype. (Left) Schematic of 3D printed casing for final prototype, with dimensions labeled in millimeters. (Right) Fully assembled final prototype on the stage of an inverted microscope.

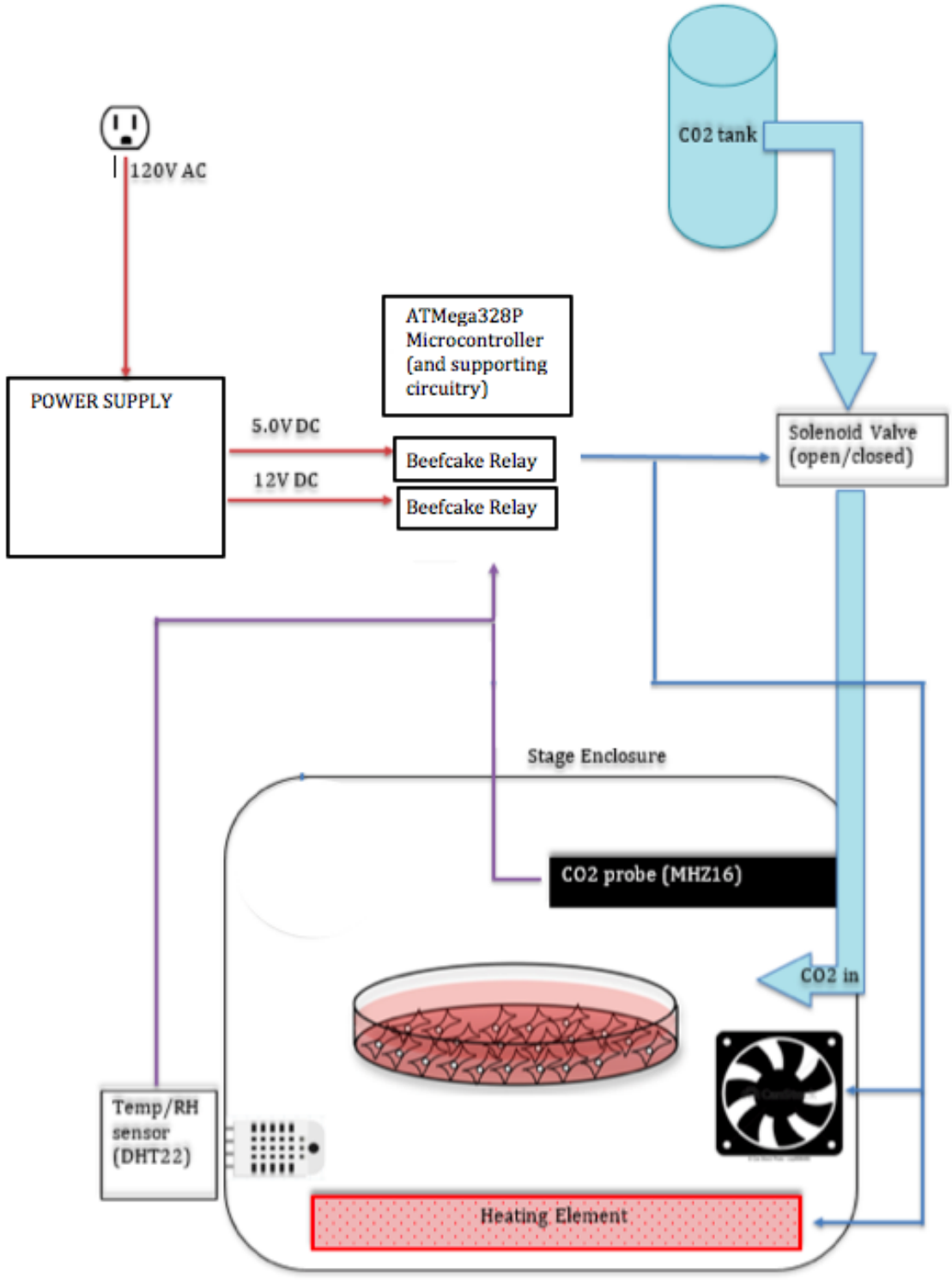

Figure 2. System diagram for the microscope incubator electronics. Red traces indicate the voltages coming from the power supply, blue wires indicate components that the microcontroller is influencing, and purple wires show feedback from the two sensors.

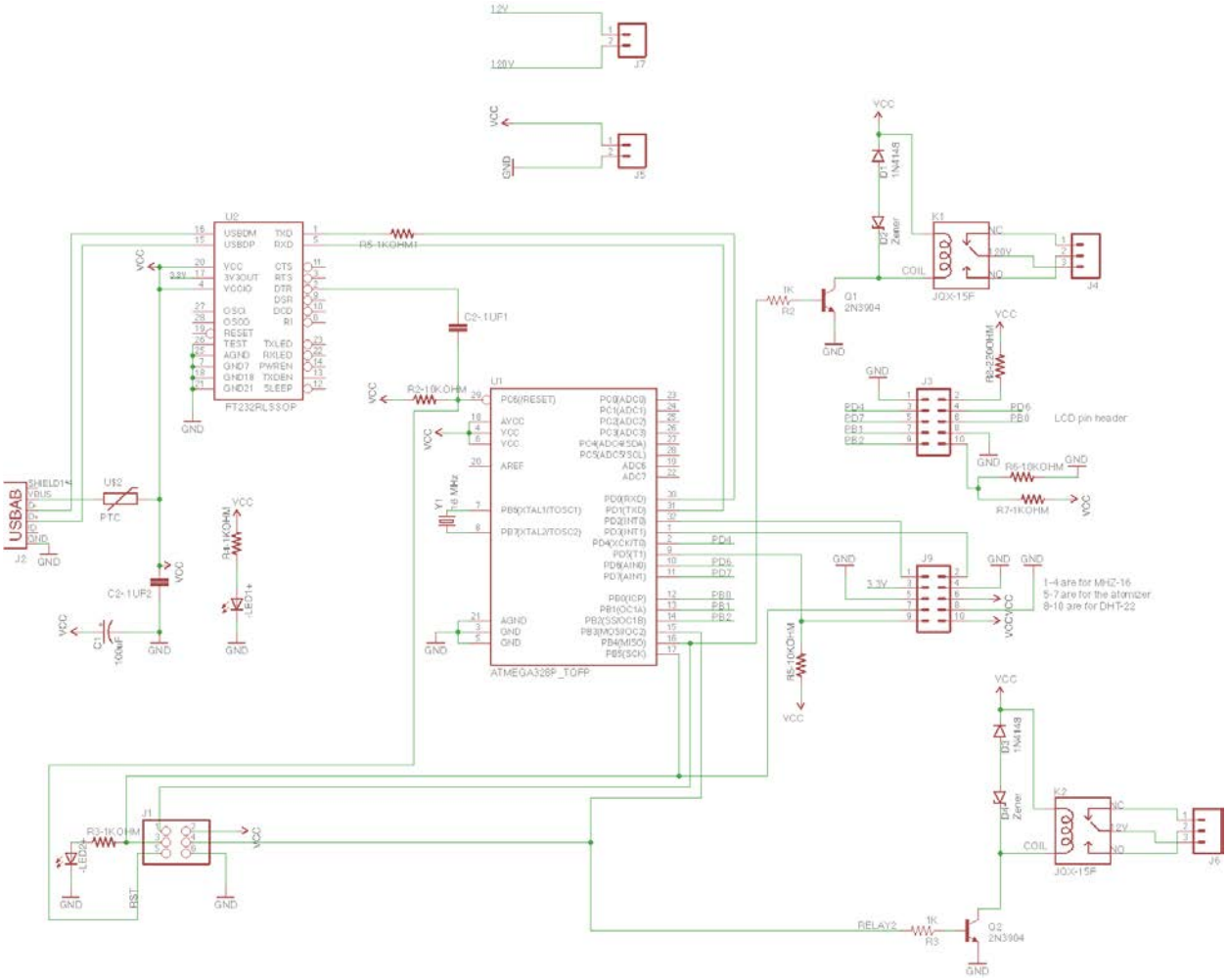

Figure 3. Circuit diagram of the PCB that was used to control the incubator. The FTDI driver and micro USB port were used to upload code, while the pin headers connected to the power supply powered the board during use. The microcontroller interfaces with the control components and LCD screen via the additional mounted pin headers

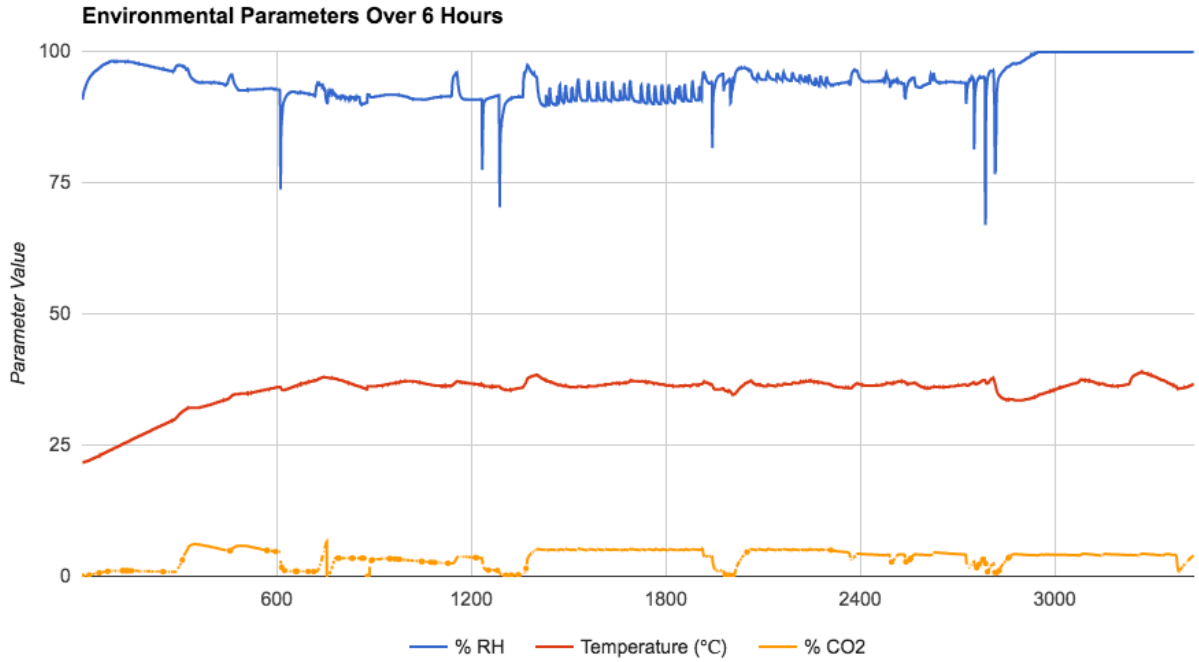

Figure 4. Temperature (red) and humidity (blue) levels in the incubator over a 90-minute duration.

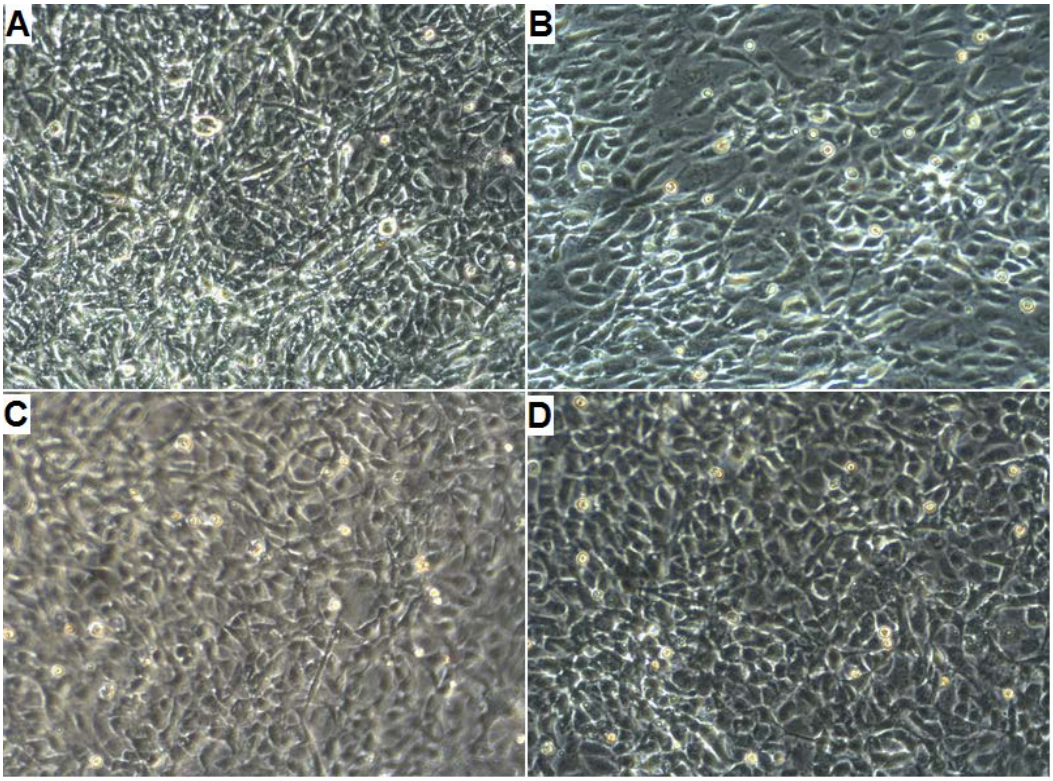

Figure 5. Results of image compatibility tests, (A) image captured through tissue culture polystyrene, (B) glass, (C) polystyrene, (D) acrylic.

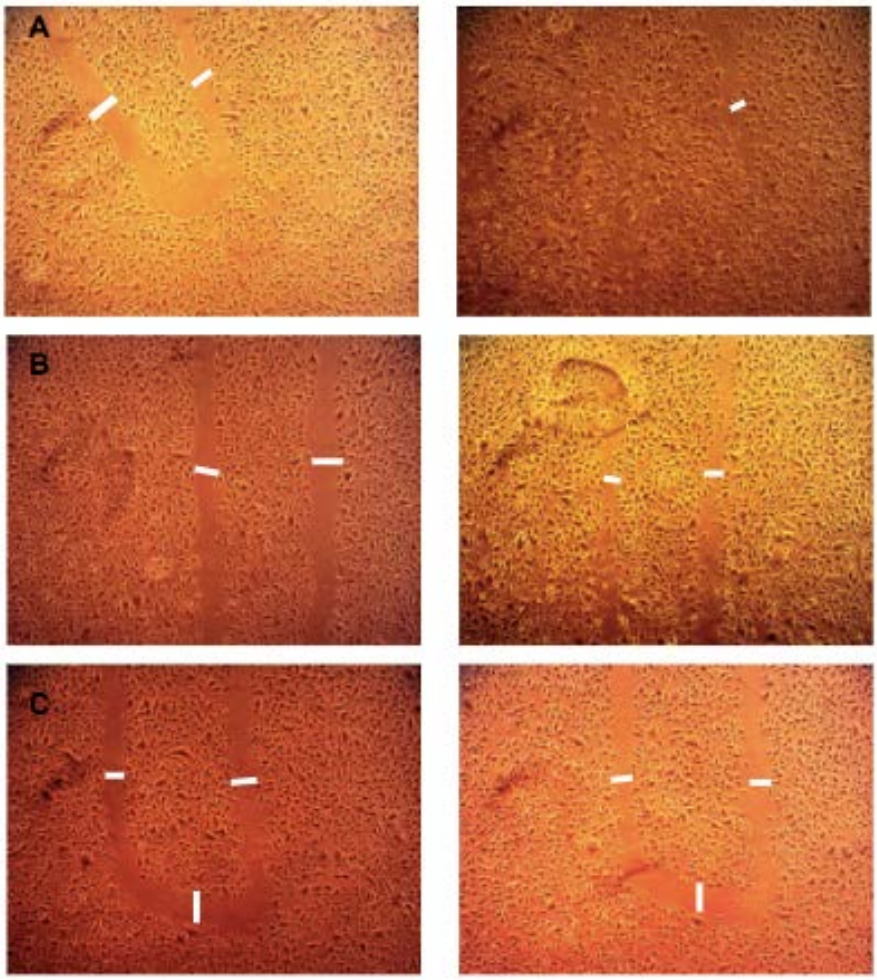

Figure 6. Scratch assay. Cells showed the much regrowth in the Sanyo incubator (A), some regrowth in the prototype incubator (B), and very little regrowth in the control (C).

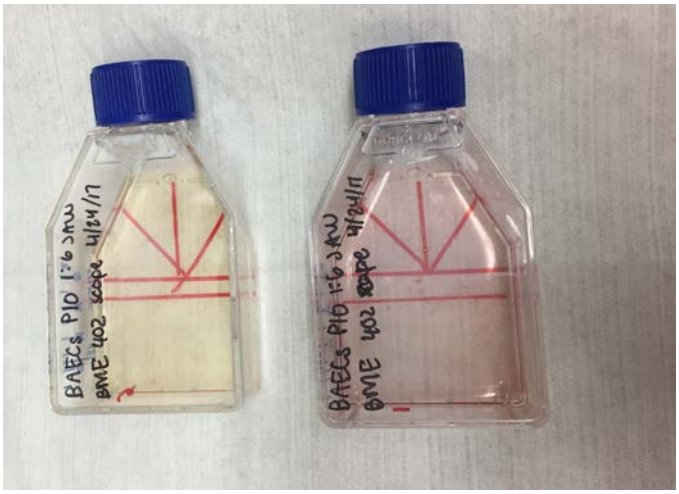

Figure 7. Media color changes after incubation. Media was more yellow after incubation in the prototype incubator (top) than the Sanyo incubator (bottom).

Tables

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

Table 1. Imaging material properties.

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 of relative image focus for various materials based on MATLAB focus measure.

Cell Culture Protocols

Cell Passaging Protocol

1. In sterile hood, aspirate media from cell culture dish(es)
2. Rinse each dish once with 3-5 mL 1X sterile PBS, then aspirate out
3. Add 4 mL (to a 10cm petri dish) 5X Trypsin, replace in incubator and let cells detach for 5 minutes
4. Look under microscope to ensure cell detachment
5. Deactivate trypsin with 4 mL (to a 10 cm petri dish) of warmed cell media
6. Pipette solution up and down on plate, rinsing off residual cells, and place cell solution in a 15 mL centrifuge tube
7. Centrifuge (with counterweight) for 5 minutes at 1000 rpm
8. Ensure cell pellet has formed at bottom of tube, then replace in hood and aspirate media without disturbing the cell pellet
9. Resuspend with the number of mL that you would like the passage ratio to be (4mL for a 1:4, 10 for a 1:10)
10. In new dishes, add 1 mL of cell suspension to each dish, and 7 mL of cell culture media

11. Label with date, initials, cell line, and passage ratio
12. Replace in incubator

Cell Refeeding Protocol

1. Add 5-10 mL (depending on cell culture dish size) of warmed media
    a. Add 5-10 mL (depending on cell culture dish size) of warmed media
    b. for 10 cm petri dish: 8 mL
    c. for 10 cm petri dish: 8 mL
2. Replace cells in the incubator