Physiological and Molecular Changes in Various Biological Organisms Cultured under Simulated Microgravity Conditions

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Kennedy Space Center

Major: Biomedical Engineering

Program: NIFS 2017 Fall Session

Date: 17 11 2017

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Nomenclature

3DC = 3D clinostat
EC(s) = endothelial cell(s)
g = gravity
hr = hour
LINC = Linker of Nucleoskeleton to Cytoskeleton
PBS = Phosphate Buffered Saline
RPM = random positioning machine
SMG = simulated microgravity
µg = microgravity

I. Abstract

Microgravity is one of the most import factors in space flight where its impact on living biological organisms is concerned. Many different ailments have been reported in astronauts such as spaceflight related osteopenia, cardiovascular concerns, and loss of eye sight. In order to understand why µg causes these issues we must understand what is happening at the most basic of biological structures, the cell. The work done in this report is a culmination of contributions made to a much larger project. The project seeks to understand how cellular physiology is changing in SMG conditions and use this knowledge to feed into a follow-up study on the genetic changes that are seen in SMG environments. Cells were imaged using confocal microscopy after 20hrs and 48hrs in a 3D clinostat called the Gravite. Lengths, widths, heights, and total cell areas were measured using an image analysis software package ImageJ. There were significant differences in lengths and widths of cell nuclei, and total area of cell coverage. The report then discusses some of the problems with the testing apparatus and how 3D printing technology may be used to create better sample holders for the 3D clinostat.

II. Introduction

Microgravity and its effects on human biology are some of the biggest challenges to overcome if human space travel ever hopes to become long duration. There are many concerning effects from radiation exposure alone when humans enter space, but a major issue still lies in the fact that the biological responses to gravitational offloading are not well understood[1]. The goals of this project are to observe changes in cell, and cell nucleus, structure in simulated microgravity conditions. Endothelial cells are well studied in microgravity research and these studies provide a knowledge base for what to expect from different cell lines. Endothelial cells are crucial members of the human vascular system, making up the innermost layer of structures like arteries and veins[2, 3]. They are responsible for regulating cell-blood exchange and are essentially gatekeepers for nutrients and waste[2, 3]. These cells are important to study because it has been observed that astronauts are 4.5 times more likely to die from heart disease than the general population[3]. Like the fibroblasts studied in this project, ECs are mechanotransducers meaning they are sensitive to forces they experience[3]. In the case of microgravity, these signal cascades can be affected due to the change of force experienced. ECs are some of the most studied cells in µg environments but unfortunately results are inconsistent

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from study to study, some say proliferation, circularity, and actin content are increased others say the opposite and this could be due to a variety of experimental inconsistencies[1].

This project seeks to determine if the results of morphology studies are dependent on variables other than μg. Chamber slides, and Ibidi slides are used to determine effects of sample containers on the morphological changes under SMG. Several different cell lines are also used, MCF10A and NIH3T3, in addition to a genetically engineered version of the NIH3T3 cell line. NIH 3T3 cells are a well-studied mouse, embryonic, fibroblast cell line that work well with this study due to their recognizable morphology. A genetically altered version of the NIH 3T3 is also being studied to determine μg effects on gene expression related to the LINC complex, a protein system that regulates mechanosensitivity in the cells. For my internship, only NIH 3T3 cells are analyzed and represented in this report. In the future of this project experiments using different cell lines will provide information on morphological differences between cell lines in SMG environments as well as the role of the LINC complex in response to SMG.

The method of microgravity simulation is another variable that is introduced. A 3D clinostat and a random positioning machine will be used to determine if there are morphological changes happening due to the difference in simulation the two machines take. A 3D clinostat rotates the samples in two axes (X and Y) at a constant velocity, such that over time the gravity vectors the cells experience sum to 0. The RPM works in the same manner as a 3DC, but is programmed to randomize velocity, acceleration, and position producing forces that still sum to 0 on the sample. Between these three variables: cell line, sample container, and simulation method, the project hopes to produce results that provide insight to the degree of uniformity that must be maintained between studies.

The confocal microscope is used for observing cell and nucleus morphological changes. Confocal microscopy is a sophisticated, state-of –the-art, imaging technology. The microscope functions by having various excitation laser light sources that correspond to the wavelength of the stains used on the samples[4]. In this case the stains are Hoechst and phalloidin to observe the nucleus, and actin content of the cells, respectively. The laser is shot at a dichromic mirror that reflects the light toward the objective lens, which focuses the laser's light on the sample[4]. There are two more mirrors, the x mirror, and the y mirror that the laser ricochets off of before hitting the sample[4]. These mirrors are placed to allow the laser to scan the sample[4]. The scanning of the sample allows the process of precise measurements and 3D image reconstruction in high resolution[4]. The light that reflects off the sample then passes through the dichromic mirror[4]. Behind the dichromic mirror is a pinhole configuration that captures only light that is focused in the current z-plane[4]. The microscope adjusts the distance between the sample and objective lens in order to take slices and reconstruct a 3D image of the cells. Using this technology to capture high resolution 3D images, ImageJ can then measure height, area, width, and length of the cells to study morphological differences.

III. Methods

The author of this paper is an intern contributing to this project and as such only the methods performed by the author are represented in this section. This section is not an exhaustive list of methods, but rather a reflection of the work the author has performed and contributed.

Thawing Procedure for NIH3T3 Fibroblasts:

1. Remove NIH3T3 cryovial sample from liquid nitrogen.
2. Place cryovial in a 37°C water bath until the vial is *mostly* thawed. Less than a minute of time should be fine.
3. Clean with 70% ethanol and place under the tissue culture hood.
4. Place contents of cryovial into a 15mL centrifuge tube.
5. Using a 10mL pipette, take 9mL of media (DMEM) and add 2 drops at a time, all the while shaking to mix. Take small breaks between drops to give cells time to adjust.
6. Once all 9mL have been added pipette up and down with 5-10 times to thoroughly mix.
7. Centrifuge at 130 rpm for 6 minutes.
8. While centrifuging label 1 T75 flask with cell type, passage 1, initials, and date.
9. Remove centrifuge tube and take back to the tissue culture hood. Aspirate to the pellet.
10. Resuspend the cells in 10 mL of DMEM.
11. Add resuspended cells to T75 flask.
12. Replace pipette and add 5 more mL of media to the flask.
13. Change media the next day to remove DMSO, and then every other day after.

All cell procedures for NIH3T3 cells are adapted from publicly available information from NIH/3T3 (ATCC® CRL1658™)

Fibronectin Application:

1. Dilute fibronectin to 50µg/uL in PBS.
2. Each chamber slide requires 1 mL of diluted fibronectin solution.
3. Each Ibidi slide needs .25 uL of diluted fibronectin solution.
4. Under the tissue culture hood, add the solution to the chamber and make sure that the solution covers the entirety of the bottom of the container.
5. Place containers in fridge overnight.

Cell Staining Procedure:

1. For every Ibidi slide that needs staining add 200uL to total. For chamber slides add 400uL.
2. After total has been determined set aside total amount of PBS.
3. In PBS dilute phalloidin, add 3uL for every 200uL of PBS.
4. To the same PBS solution add 1uL of Hoechst die for every 1000uL of PBS.
5. Add respective amounts of solution to all samples and make sure the bottom of samples are coated.
6. Let stain sit for 15 minutes.
7. Aspirate dye using manual pipettes.
8. Wash with PBS 2-3 times.
9. Fill Ibidi slides with PBS.
10. Seal Ibidi slides using parafilm and place in -20°C freezer.
11. Remove chamber portion of chamber slides.
12. Add two drops of gold anti-fade mounting media.
13. Carefully align and place a glass coverslip over mounting media and sample.
14. Cover chamber slides with aluminum foil and allow drying for a day.

All cell procedures for NIH3T3 cells are adapted from publicly available papers relating to NIH/3T3 (ATCC® CRL1658™)

Image J Cell Nucleus Length and Width Measurement:

1. Open the .N2 file saved from the confocal microscope as a hyperstack and select split channels.
2. There should be two images. The blue image is that of the nuclei, scroll if both images are black.
3. Click on the nuclei image and click *Image - Stacks - Z Project*.
4. Click *Image – Type – 8 bit*.
5. Click *Image – Adjust – Threshold*.
6. Select *Dark Background* and manually adjust the threshold until the entirety of the nucleus is covered without adding any additional aspects to the nucleus due to noise. Usually it is ok to have a few holes with a crisp edge because the software can fill holes.
7. Click *Analyze – Set Measurements – Fit Ellipse*.
8. Click *Analyze – Particles – Outlines – Include Holes – Display Results*.
9. Create an excel sheet and save the minor and major ellipse measurements as length and width. Be sure to keep the nomenclature consistent (always call the bigger side length).

Image J Cell Area Measurement:

1. Open the .N2 file saved from the confocal microscope as a hyperstack and select split channels.
2. There should be two images. Select the red image that stains actin to see total area of the cell.
3. Click on the actin image and click *Image - Stacks - Z Project*.
4. Click *Image – Type – 8 bit*.
5. Click *Image – Adjust – Threshold*.
6. Select *Dark Background* and manually adjust the threshold until the entirety of the cell is covered without adding any additional aspects to the nucleus due to noise. Usually it is ok to have a few holes with a crisp edge because the software can fill holes.
7. Click *Analyze – Particles – Outlines – Include Holes – Display Results*.

9. Create an excel sheet and save the area measurements.
The ImageJ macro created for length and width measurement can also be used for area measurements so long as it is on the red actin image.

Image J Cell Nucleus Height Measurement:

1. Open the .N2 file saved from the confocal microscope as a hyperstack and select split channels.
2. Crop the blue picture so that no cells are positioned "on top of" one another.
3. Click *Image – Stacks – Reslice* be sure the slices are set to 0.3 microns.
4. Click *Image – Z Project – Max Intensity*.
5. Draw straight line over the nucleus, make sure the line is in a uniform intensity area, chromatin can cause bright spots.
6. Click *Analyze – Plot Profile*, optimal profile will have one peak and both ends will taper to 0.
7. Click *Select List.*
8. Save as a .csv file with filename that identifies the sample, cell line, and cell.

Creation of ImageJ Macros:

1. ImageJ macros are easy to make using the record feature in *Plμgins – Macros- Record.*
2. Once recording has begun go through the steps of your image analysis procedure and make sure ImageJ is automatically recording the steps.
3. Once the procedure is complete, the code is generated and can be copy and pasted into ImageJ's startup macros.

Power Analysis:

Power analysis was run after preliminary results showed significant differences in length measurements of cells in microgravity conditions for 20 hours and controls. Rstudio's power library was utilized to run the power analysis. The function "power.t.test" was run and below are the variables that are passed to the function:

Delta: The difference between the means of interest, control and test condition.
Sd: Standard deviation of the control
Sig.level: The significance level the experiment is aiming for.
Power: 1-sig.level
Type: The type of t test being run.
Alternative: Specifies a two tailed or one tailed test.

IV. Results of Size Measurements

Size measurements are performed using the methods in ImageJ described in the *Methods* section. Figure 2 shows images of cells in two planes, the x-y plane, and the x-z plane. The former plane is used to take measurements of length and width and the latter is used for measurements of height. The left cells of each picture are control cells that grew normally in a 1g static environment for the same amount of time as the SMG cells. Though there are only 4 cells shown in the figure over 40 were analyzed for each condition as determined by power analysis.
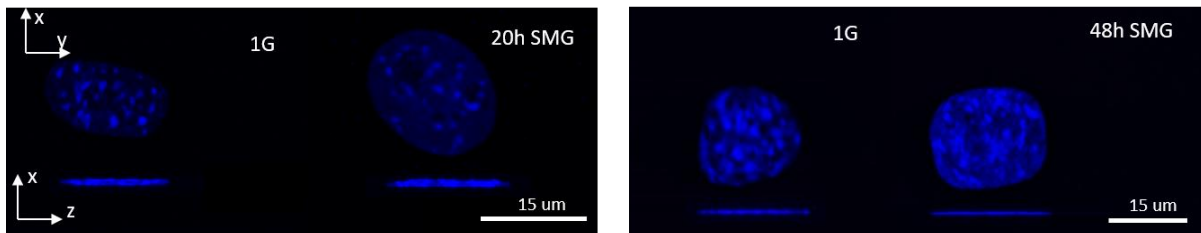
Figure 1. Images taken of control cells and 20hr simulated microgravity cells on the left. On the right is a control cell and a 48hr simulated microgravity exposed cell.

Figure 3 shows the statistical results of a t test for differences in length measurements for 20hr, and 48hr SMG. All error bars are representative of the standard error of mean. Statistical differences were found between both the controls and both the time conditions. This means that SMG does have an impact on how the cells grow in length. One important aspect to note is that the difference in the 20hr condition is greater than the difference in the 48hr condition. This might be due to some adaptation that the cell makes over the 48 hour time frame. Or in 48 hours more cells are able to reach a G0 phase and are not dividing, or changing size in preparation to divide. Previous studies suggest that proliferation is effected by a μg environment but are not consistent with whether or not there is a positive or negative correlation[2]. Figure 4 shows that the width of the cells only change significantly in the 20hr condition. This could be due to multiple factors such as proliferation, growth signaling pathways, etc that cannot be determined from these results alone.
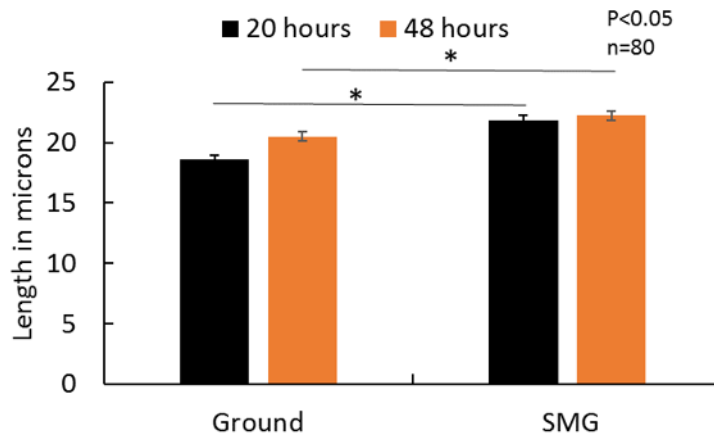
Figure 2. **The differences in length between SMG cell nuclei and control cell nuclei at 20hr and 48 hr time-points**. **Significance was found between the 1g and SMG conditions for both time-points.**
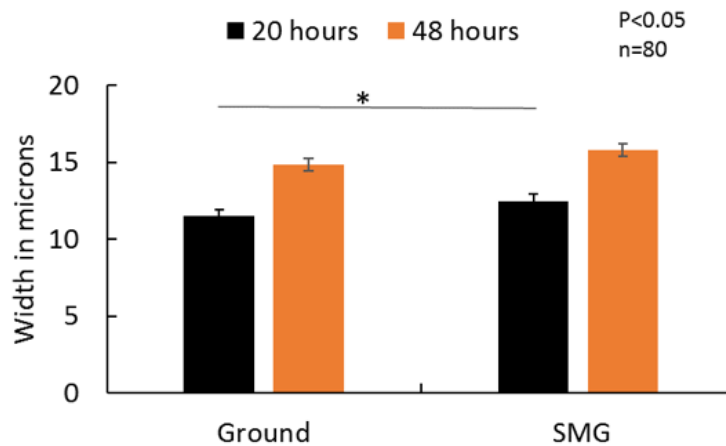
Figure 3. **The differences in width between SMG cell nuclei and control cell nuclei for 20hr and 48 hr. Significance was only found in the 20hr group but not in the 48hrs group.**

After examining nucleus lengths and widths, total cell area was measured using ImageJ software. Figure 5 illustrates the observable difference in size between cells in each of the different conditions. Consistent with the results of the t test in fig. 6 the cells on the left represent control cells and the cells on the right are SMG cells of 20hr and 48hr exposure. Figure 6 shows that there was statistical significance between the cells cultured under controls and SMG environments.
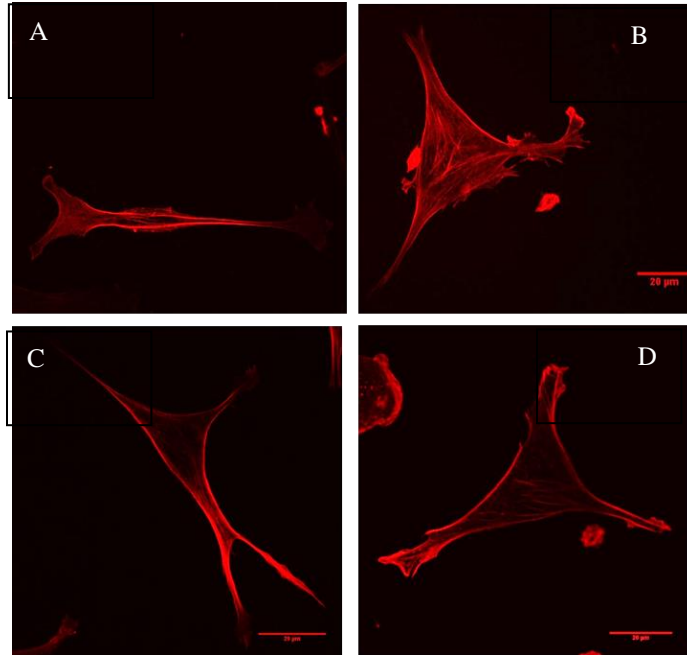
Figure 4.   Images of NIH3T3 Fibroblasts taken using confocal microscopy. The red dye is the phallodin that binds to actin in the cells. A) Control 20hr, B) SMG 20hr , C) Control 48hr D) SMG 48hr.
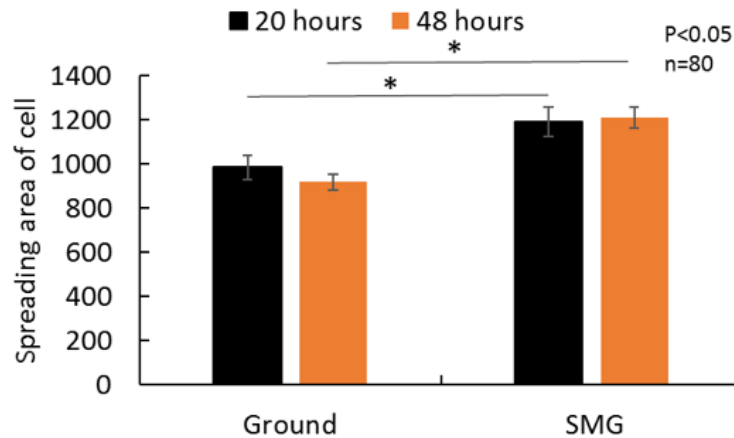
Figure 5.   The differences in total cell area covered of cells cultured under controlled and SMG conditions 20hr and 48hr time-points.

V. 3D Printing Designs

An important side project was the development of sample holders for the Gravite 3D Clinostat to secure the cell culture containers used for the study onto the Gravite stage. The 3DC came with its own sample holder, but the holder was only suitable for T25 flasks and not the chamber or Ibidi slides that are being tested. Due to this, samples were held in place with webs of tape that made removal of samples difficult, and placing of samples caused dips in incubator temperature and CO_2 levels due to the amount of time the door was open. Four concept models were developed in Solidworks for use with the Gravite 3DC. The first of these concepts was a simple plate seen in Fig. 7. The idea being that the screws on the base plate could be replaced with longer screws. Once they were replaced, they could feed up through to the 3D printed plate and the height could be adjusted to snuggly fit the sample. This became a problem since this initial concept could not hold the Ibidi slides. The issue was that the Ibidi slides have two portions that protrude upward so the slides would only be held in place by these two points of contact rather than by a full face area of contact. The two protrusions are also the location of sample loading and applying pressure to them may introduce unwanted pressure to the samples.
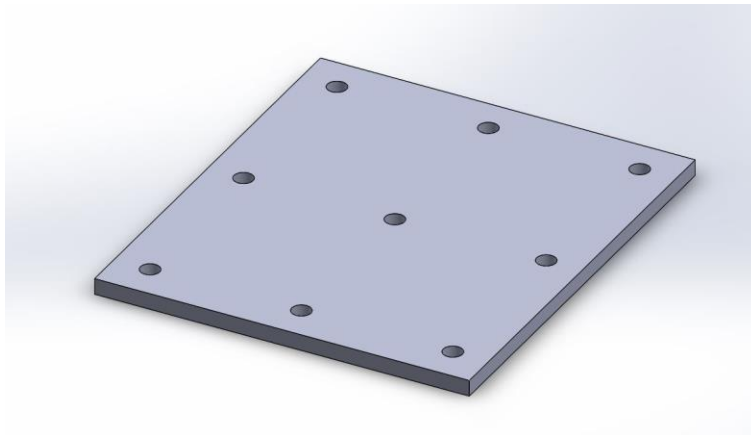
Figure 6. The initial concept for a sample holder.
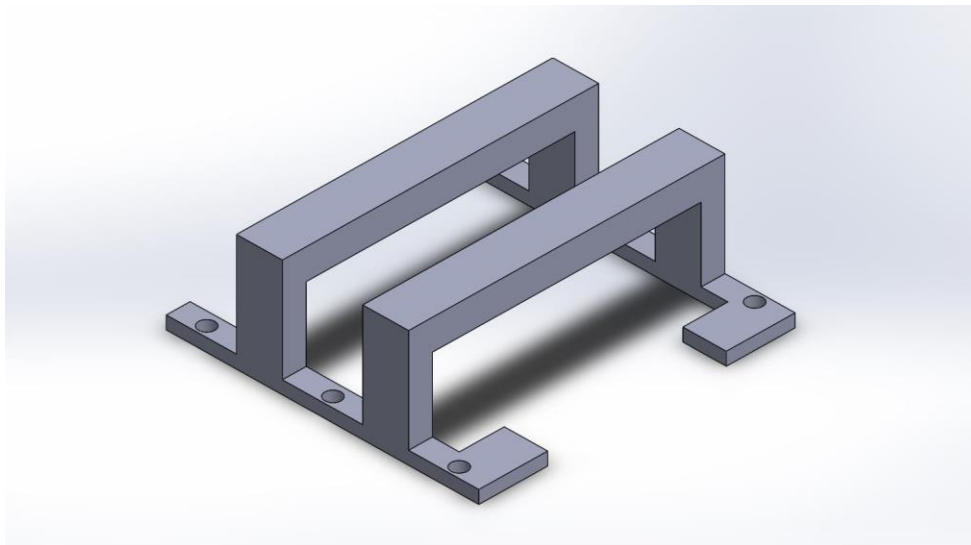
Figure 7. The second concept for a sample holder.

Concept 2 (Fig. 8) was created with being a secondary holder in mind. Foam blocks may be created and shaped to fit different samples and sample combinations. These foam blocks would slide into concept 2 and snuggly secure the samples for the duration of the simulation run. Figure 9 shows how samples, independent from the foam blocks could

also be secured using this holder. The downside of using samples without the foam blocks is that the minute differences between sample plastic could be the difference between snuggly staying secure and falling out of the holder since the height is an important factor. Ideally, all foam blocks that were used would be the same height so this issue is not a problem in the intended usage of this concept. This concept was not tested due to the lack of foam holders needed for the testing. This bracket is more of an idea of what could work in future iterations of sample holders.
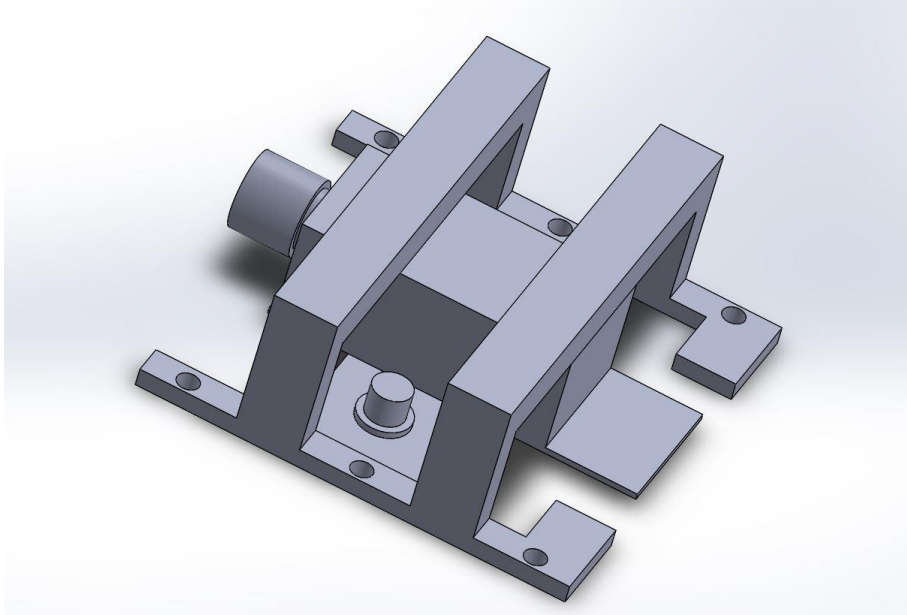
Figure 8. A representation of the second concept in action.

The third concept was made with simplicity in mind. Pictured in fig. 10 it looks a lot like the typical bathroom plunger. Figure 11 demonstrates how the holder actually holds a sample in place. A web of rubber bands is placed on the base plate and the plunger acts as a handle to pull the web upwards to place the sample in. Once lowered back down the plunger head provides a clamping point for the web to secure the sample(s) in place. This design is beneficial because the rubber bands are flexible enough to hold most sample configurations and still provide the force necessary to keep the samples suspended. This concept was tested and worked surprisingly well. The only sample configuration that this holder did not work on was a configuration that used two chamber slides. In fig.11 one chamber slide can be seen, but when two are placed near each other they create a gap that the plunger cannot bridge. This makes it difficult for the force of the rubber bands to be focused on the samples and causes the samples to slip loose. Figure 12 shows the largest sample load that the plunger can hold in place after testing.
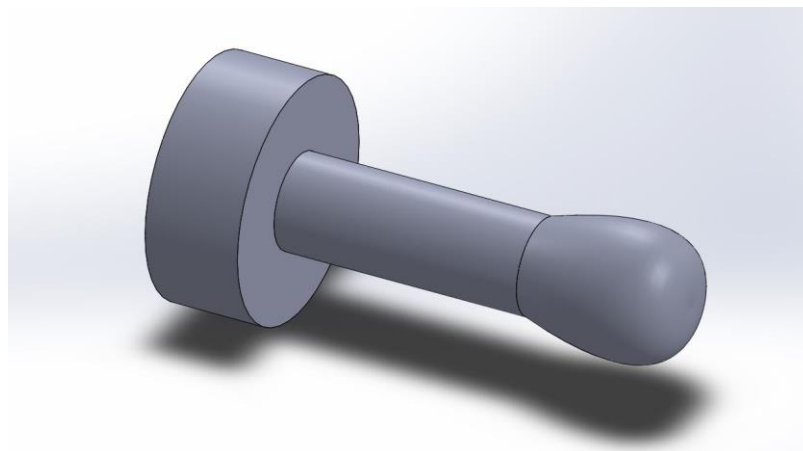
Figure 9. The third concept for the sample holder.
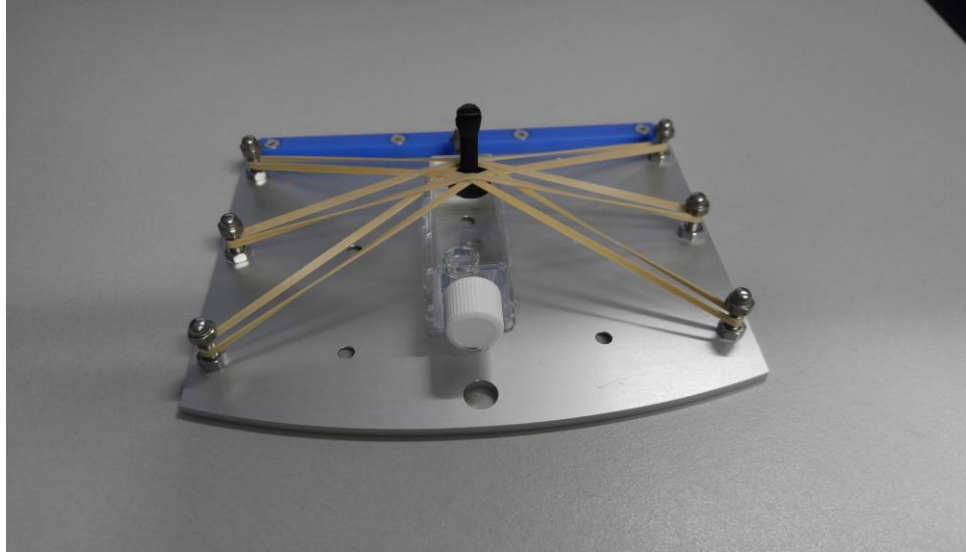
Figure 10.   **The "plunger" concept in action.**
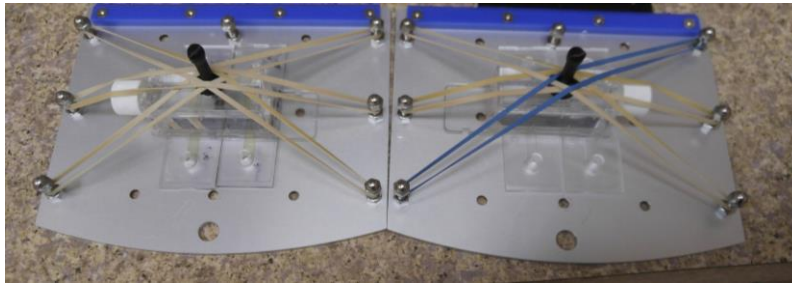
Figure 11.   **The "plunger" concept holding a max sample size of one chamber slide and two ibidi slides.**

The final concept, the "X", was created to fix some of the problems with the initial concept. With the X design, samples can be placed easily and will always interface in large areas so that forces will be dispersed and no pressures will build. The "X" design is an improvement on the initial concept but should be reserved for heavy sample configurations that the plunger design may not be able to tolerate. Figure 13 is a model of the concept and fig. 14 shows how the concept would be configured with samples. When tested this concept works well. The only problem with the "X" design is that it can be time consuming to get the samples on. The "plunger" is able to load samples in approx. 10 seconds. The "X" design can take up to 30 seconds depending on how used to the hardware the user is. This design can be seen mounted in fig. 15. In the future wing nuts will be used instead of normal nuts to make it more ergonomically friendly.

Figure 16 displays all of the concepts and the base plate to demonstrate relative sizes. The base plate (left) is approximately 5 inches wide by 6 inches long and 1 tenth of an inch thick. Figure 17 is a table that I created that shows test results for the different concepts and recommends which concepts can be used in which sample situations. According to the results, the "plunger" is the concept that should be used for most sample configurations. The "x" concept can do everything the "plunger" can but requires hardware that takes time to attach properly and has the potential of wiggling loose. The only case in which the "X" should be used over the "plunger" is in the case of multiple chamber slides since they are heavy. This could be fixed by using stronger rubber bands or modifying the bottom of the "plunger" to have a wider area of interaction and coating it in rubber. Stronger rubber bands could also be used to clamp the samples better
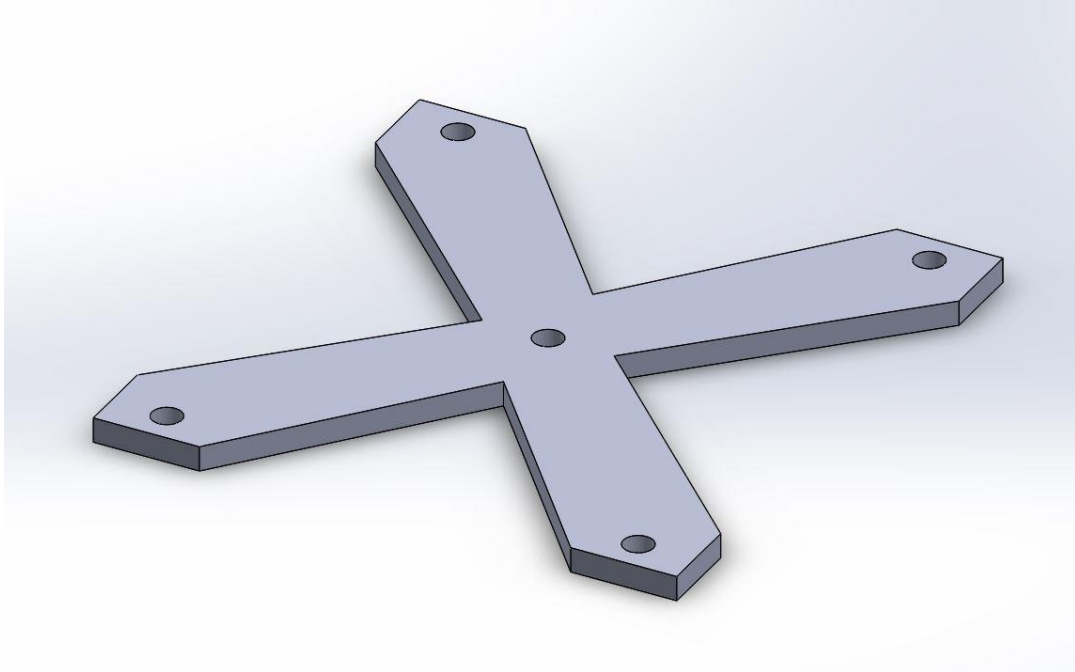
Figure 12.   The fourth concept for the sample holder. The "X" concept.
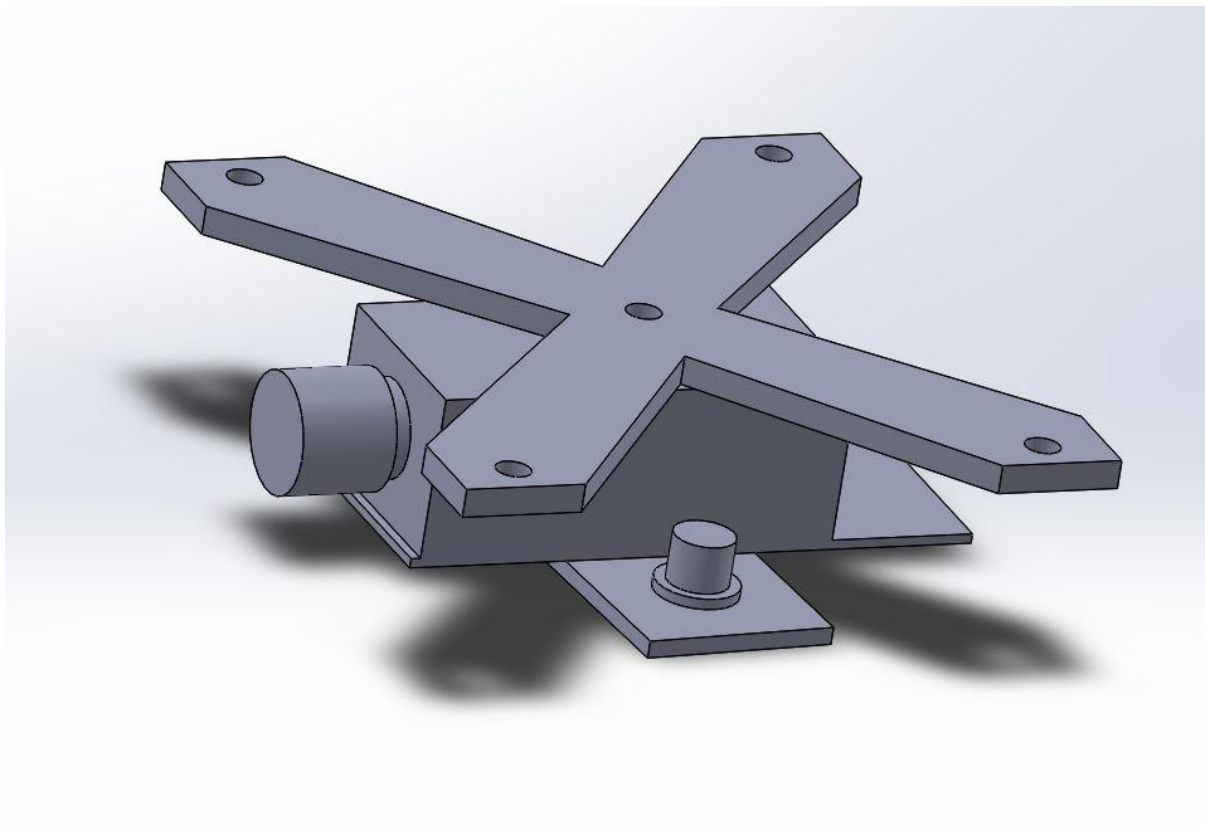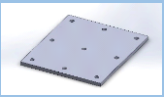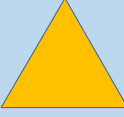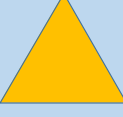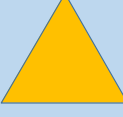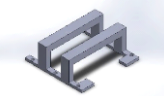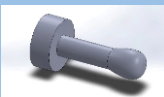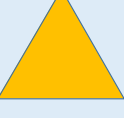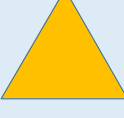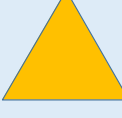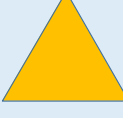
Figure 13.   A representation of the fourth concept in action.
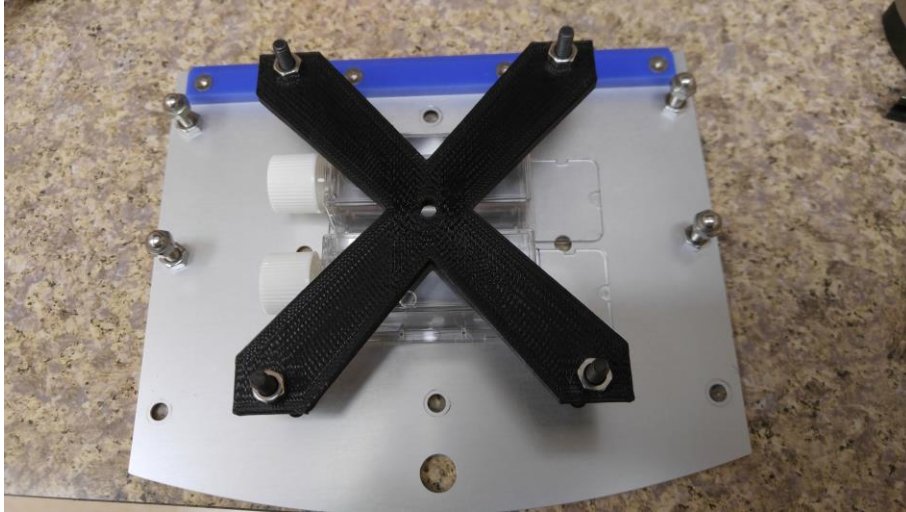
Figure 14. The "X" concept mounted to the base plate of the Gravite. This concept is best used when multiple chamber slides need to be held by both base plates.
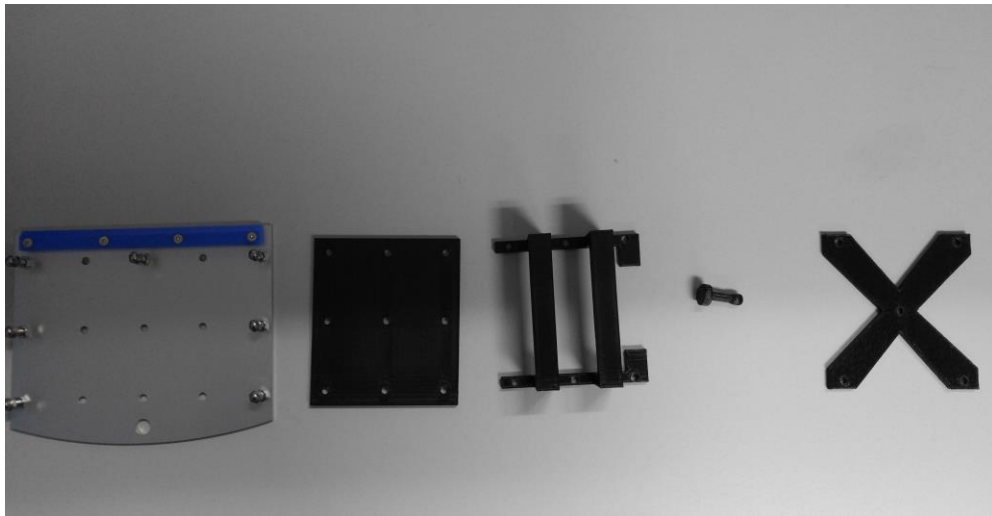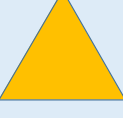

Figure 15. 3D printed versions of all concepts next to the plate they mount on.

Concept Sample Test Configurations Pass/Fail	1 Ibidi Slide (IS)	1 Chamber Slide (CS)	2 Ibidi Slides	2 Chamber Slides	1 CS + 1 IS	1 CS +2 IS
Concept 1: Plate	✖	🔺	✖	⭕	🔺	🔺
Concept 2: Bracket Holder	Not tested due to the need for foam blocks. This concept was a holder for foam and would fail the sample tests.					
Concept 3: Plunger	⭕	⭕	⭕	✖	⭕	⭕
Concept 4: X	🔺	🔺	🔺	⭕	🔺	🔺
Pass Criteria: Did the samples stay in place for 24 Hours?						
Legend	⭕ Passed Test and Recommended		🔺 Passed Test but not Recommended		✖ Did not pass test and not recommended	

Figure 16. A table created based on testing results of the various concepts. The "plunger" did the best overall being very easy to use and holding the samples in place.

To fix the issues experienced with the "plunger" design and larger sample loads, the base was made larger and thicker. This change increased the area of contact between the "plunger" and the chamber slides and helped disperse the force of the rubber bands between the two samples. After these modifications were made the "plunger" was able to successfully hold the samples through testing. Figure 18 shows the model of the newest "plunger" design.
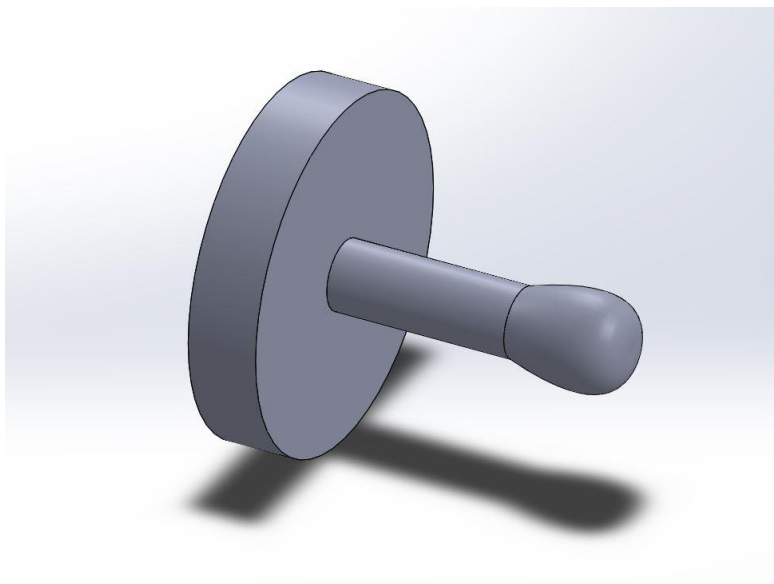
Figure 17. An updated version of the plunger concept. The base has been expanded to better disperse the forces on larger samples.

VI. Conclusion

My time at NASA has been fun and educational. I am leaving with a greater respect for the research that goes on behind the scenes to make human spaceflight possible. My work on the project added data that will be used to gain a better understanding of SMG and its comparison to actual µg environments. It will also help when the project moves into its next phase of studying genetic markers to see what causes the observed physiological changes. I am sure that the work I provided on the sample holders will make experiments more streamlined and easier to perform. The work performed here is important in determining variables that are essential to establishing uniformity and consistency in future µg studies.

Acknowledgments

I would like to thank Dr. Ye Zhang, and Dr. Srujana Neelam for being exceptional mentors and very supportive of my contributions to the project. Thank you to NIFS for making this opportunity possible and to all the individuals I have met in the UB department who were kind, welcoming and patient.

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