Lab Report #1

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Abstract*— Mouse fibroblasts, also known as 3T3 cells, were used to test the effectiveness of different substrate coatings on PDMS, and the biocompatibility and toxicity of different glues for PDMS. Cells were sub cultured using DPBS (-), trypsin, and complete media (10% FBS). For substrate coating, type 1 bovine collagen, gelatin from porcine skin, poly-L-lysine (0.01%, 0.1%), poly-D-lysine (0.1 mg/mL), poly-L-Ornithine, dopamine hydrochloride, and poly-HEMA were tested on 24-well non-tissue culture plates. 250 µL of each coating solution was put into their respective wells, incubated, aspirated, rinsed, and then air-dried. Then 500 µL of cell suspension was added to each well with the positive control being 35 mm plate. These were incubated and were imaged. To test biocompatibility, 1 well in a 12 well plate was used for each material. Acrytol, cytoseal, rubber cement, silicone glue, scotch double sided-tape, nail polish, liquid band-aid, and gorilla super glue were tested; the left-over wells were used as control. After UV sterilization, 1 ml of cells were seeded into each well. They were incubated, then imaged. After 7 days, 0.01% poly-L-lysine (PLL), collagen, gelatin, poly-D-lysine(PDL), and poly-L-Ornithine(PLOR) were found to have healthy and attached cells. Other coatings caused cells to clump or create sheets of cells. Acrytol and tape were found to have healthy cells, proving that they are not cytotoxic. The rest of the adhesives seemed to be less effective or even cytotoxic. For future use, gelatin, collagen, PDL, PLOR will be used to coat PDMS with acrytol as the adhesive to the plate as these materials proved to be the most effective as they allowed for healthy attached cells and clear imaging.

I. Introduction

Cell culture is the growth of cells under controlled conditions. This process is normally done in a laboratory, not in a natural environment. Cell culture is widely used in pharmacology, toxicology, oncology, virology, cytology, and biomedical engineering. In the field, it is used to study model systems, drug screening, and more. The sources of cells can either come from primary cells or cell lines. Primary cells are directly from a patient and normally have a limited life span while cell lines are from normal cells that were transformed spontaneously in cultures. Cell lines continue to replicate beyond the Hayflick limit. Primary cells are preferable as it is easier to control and don't go over the Hayflick limit. An important factor used in culturing cells is complete media. Complete media consists of a basal medium (like DMEM), fetal bovine serum (FBS), L-Glutamine or glutamax, non-essential amino acids, vitamins, and antibiotics. DMEM contains essential salts, amino acids, essential vitamins, sodium pyruvate, glucose, sodium bicarbonate (buffer agent), and phenol red (pH indicator). Sodium pyruvate is important for energy metabolism and the pH indicator helps to see if cells are still healthy. The optimal pH is 7.2-7.4 which is a red color. Sodium bicarbonate, the buffering agent, helps to stabilize the pH. The fetal bovine serum comes from fetal calves - containing proteins, growth factors, and hormones - and helps cells proliferate. L-Glutamine or glutamax is used for energy production, protein synthesis, and nucleic acid synthesis but they are broken down differently by cells. L-Glutamine generates ammonia as a by-product, which is toxic to cells and affects protein glycosylation. Glutamax is broken down after cellular uptake which is more stable for solutions. Antibiotics prevent contamination like bacteria, yeast, and fungi to grow in cell culture plates. Penicillin and streptomycin are antibiotics that control the growth of contamination. To continue to prevent contamination, techniques like sterilization with 70% isopropyl alcohol and UV light are used. The isopropyl alcohol is used to spray anything that goes under the hood/biosafety cabinet while UV lights treat plates and dishes.

Sub culturing of cells is the transference of cells from one culture vessel to another which allows further expansion and testing of cells. General guidelines are to choose the right size of the culture vessel and seeding density. The frequency of passaging cells depends on the experiment and the laboratory; some laboratories ask for once a week and others may ask to passage the cells every day. Something that is kept in mind when passaging and subculturing is cell age, population doubling, and Hayflick limit. Population doubling (PDL) is the age of cells and cell lines. For primary cells, they have a finite life span which can be seen through the cumulative population doublings. Population doubling levels is the number of times cells divide and double; once cells stop dividing, that means it hit the Hayflick limit. It's important to remember the Hayflick limit as that signifies whether cells are still viable and can still proliferate. PDL can be found by cell counting before replating/passaging the cells. Hemocytometers can be used under a microscope to count the estimated average amount of cells in the plate. 7 µl samples are taken from the culture and put inside the hemocytometer. Consistent numbers allow for a consistent PDL. Some factors that can affect cell counting are the size of the sample in the chamber, dilution of cells, and proper mixing of the cells. Other factors to consider when passaging cells are the pH,

temperature, tonicity, and humidity. As mentioned, the optimal pH for cells is 7.2-7.4. This is possible because of the phenol red in the culture media. If the cells are in the optimal range, they will be red in the culture dish. If there is higher confluency, it may also be red-orange or orange-yellow. If the cells are below 6.8 pH, the phenol red will turn yellow and if the pH is 8.2 or higher, it will turn pink. Mammalian cells are kept at 37°C to induce cell growth and oxygen solubility. The relative humidity is 95% to prevent evaporation of the complete media and to maintain the tonicity of the media.

II. Materials and Methods

A. Subculturing

3T3 fibroblast cell lines (ATCC, CTL-1658) were cultured in complete media composed primarily of DMEM basal media (VWR, 45000-316) and supplemented with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals, S11150), 0.5 ml glutamax (Invitrogen, 35050-061), and 0.5 ml Penicillin/Streptomycin (VWR, 12001-692). The cells were cultured for nine days and sub-cultured in four passages at approximately 95% confluency. Passages occurred between 2-5 days after seeding. Before the use of the biological safety cabinet, each team member followed appropriate sterilization procedures such as using 70% isopropanol to clean. First, each team member observed their original cell culture under the microscope for any contamination then continued to work under the hood. The team aspirated any media in the dishes, rinsed the plates with 5 ml of 1X DPBS (-) (VWR, 45000-434), and aspirated once again. Sub-culturing was performed by trypsinizing the cells by adding 3 ml of 0.25% Trypsin-EDTA (VWR, 45000-664), incubated on the slide warmer at 37℃ for 7 minutes. Trypsin was inactivated by adding 2 ml of the appropriate complete media, pipetted vigorously, and spun at 206 RFC for 7 minutes in a fixed angle rotor centrifuge (Walter Products Inc). For the first three passages, the team resuspended cells in 10ml of complete media, and 1 ml was plated. In the last passage, cells were resuspended in enough of the complete media to obtain $1x10^6$ cells/ml (1000 cells/µl). Team members then seeded 250,000 cells (250 µl). The cells were observed under an inverted phase-contrast microscope (Carl Zeiss Suzhou Co, Primovert) imaged and analyzed.

B. Cell Counting

Each team member collected 7 µl of resuspended cells in a C-Chip disposable hemocytometer (Bulldog Bio, DHC-N01). Using an inverted phase-contrast microscope (Carl Zeiss Suzhou Co, Primovert), team members counted the number of cells in 4 corner squares each a volume of 1/10,000 ml. Each team member counted from the top left corner square in each of the larger squares and counted within all 16 smaller squares.Team members counted the average amount of cells in 1/10,000 ml.

C. Coating

The team used 8 different coating materials throughout the experiment with 2 wells of a 24-well non-tissue culture plate (Celltreat, 229124) for each material. The coatings the team used included Bovine collagen Type 1 PureCol EZ Gel (Advanced Biomatrix, 5074-35ML), Gelatin from porcine skin (Millipore Sigma, G1890-100G), Poly-L-Lysine solution (0.01%) (Millipore Sigma, A-005-C), Poly-L-Lysine Solution (0.1%) (Millipore Sigma, P8920-100ML), Poly-D-Lysine solution (0.1 mg/mL) (ThermoFisher, A3890401), Poly-L-Ornithine powder (5mg) (Advanced Biomatrix, 5172), Dopamine hydrochloride (Millipore Sigma, H8502-25G), Poly-HEMA (Millipore Sigma, P3932-10G). 2 wells were also used as a negative control with no coating. A 35 mm tissue culture plate with no coating was used as the positive control. 250 µl of each coating material was added to the corresponding wells. The team incubated the 24-well plate at 37 ℃ for an hour in the incubator. After incubation, the team aspirated all solutions under the biosafety cabinet. The team rinsed and aspirated each coating with MilliQ water twice. Then the team air-dried the coatings for 30 minutes. The team added 500 µl of cell suspension to each well and the 35 mm plate positive control. Every plate was incubated for 30 minutes at 37 ℃ in the incubator for the cells to attach to the plates. Then the team imaged one well per coating using the same inverted phase-contrast microscope. After the initial images, the team placed all of the plates into the incubator at 37 ℃. The team imaged the plates 2 and 7 days after the seeding.

D. Biocompatibility

The team used 8 different adhesive materials throughout the experiment with 1 well of a 12-well Multiwell Flat-bottom Plate (VWR, 62406-164) for each material. The adhesives the team used included Acrytol Mounting Media (EMS, 13518), Epredia Cytoseal Mountant XYL (EMS, 18009), Non-Wrinkle Rubber Cement (EMS, 72170), BAZIC Silicone Glue 3.38 Oz, Scotch double-sided tape, Nail polish, Liquid band-aid, and Gorilla super glue. The team used a pipette tip to apply 2 drops of the adhesive material to one side of each well and spread it to cover a small area. Two wells were left empty as control. After applying the glue, the team sterilized the plate by applying UV radiation for 20 minutes. After sterilization, the team brought the plate into the biosafety cabinet and seeded 1 ml cell suspension into each well. Team members incubated the plate in the incubator for 20 minutes at 37 ℃ for the cells to adhere to the plate. The team imaged all the wells using the inverted phase-contrast microscope after incubation and then two days after the initial cell seeding.

III. Results

A. Subculturing

All team members conducted four subculture passages using the 10% FBS supplemented complete media. The passages occurred 2-5 days after the previous seeding. Before each subculture procedure, the team observed the plate out of the incubator. The team found the media to have a reddish/orange tinge, smooth, no floating stuff, and no bubbles. Then the team used the inverted phase contrast microscope to observe the cells' confluency. Healthy growing cells' confluency increased from the previous date.

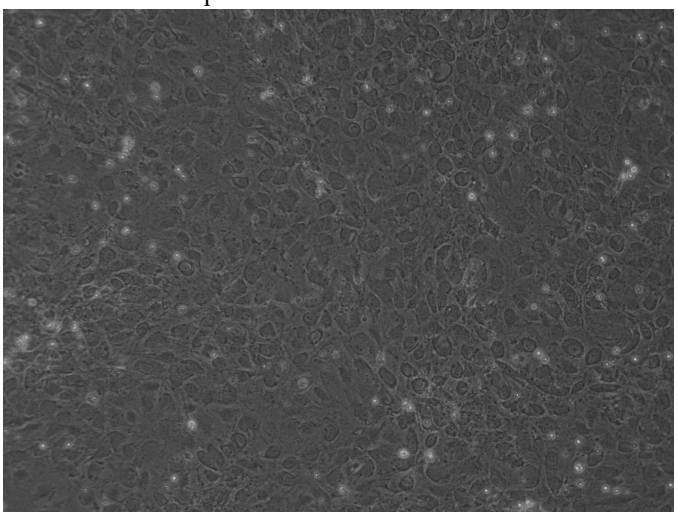

Figure 1: High Confluency 3T3 Cells. The figure above represents a culture of 3T3 cells after 5 days of incubation. The cells are highly confluent (about 95%).

After seeding, team members used the same microscope to take images of each plate.

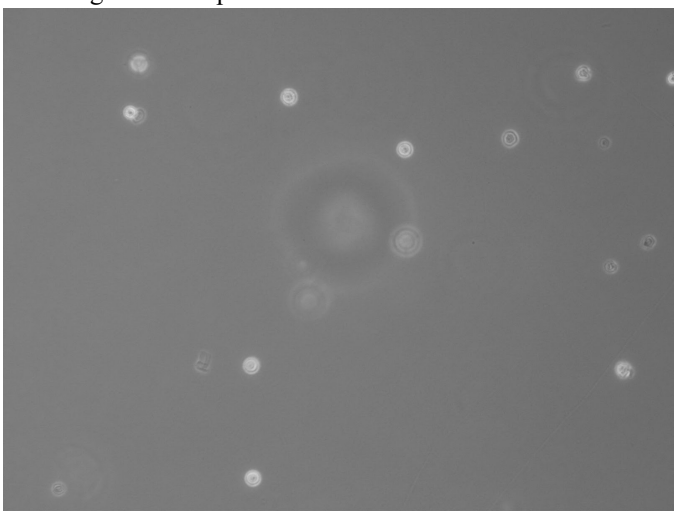

Figure 2: New Seeding of 3T3 Cells. The figure above represents newly seeded 3T3 cells after a successful subculture passage.

B. Cell Counting

Team members averaged the number of cells per square of 1/10,000 ml through the calculation

$$\frac{(Cells\ in\ Square\ 1 + Cells\ in\ Square\ 2 + Cells\ in\ Square\ 3 + Cells\ in\ Square\ 4)}{4}$$

Then, the team found the cell numbers per ml through the equation

$$Cell\ count/ml\ of\ suspension\ =\ Average\ cell\ count\ per\ square\ *\ dilution\ factor\ *\ 10,000$$

The samples were not diluted before counting, so there is no dilution factor to consider. The total cell count was determined by multiplying the number of cells in 1 ml by the total volume.

C. Coating

The team imaged wells of the 8 different coating materials 2 and 7 days after the initial seeding. With each of these images, the team made observations relating to how the cells were growing on each well. Each coating consisted of 2 different wells and the team observed both.

Coating Material	Observation
Control Positive	~50% confluent
Control Negative	Growing healthy
	Not sticking to plate, clumping together
Col	Growing healthy
	Growing healthy
Gel	Growing healthy
	Growing healthy
PLL (0.01%)	Beginning to grow
	Very confluent
PLL (0.1%)	Not sticking
	Not sticking
PDL	Starting to stick
	Beginning to grow
PLOR	Highly confluent
	Highly confluent
DOP	Slightly confluent
	~50% confluent

HEMA	Large clumps, not growing
	Large clumps, not growing

Table 1: Coating Observations Day 2. The table above represents the observations the team made for each of the coating materials the second day after initial seeding.

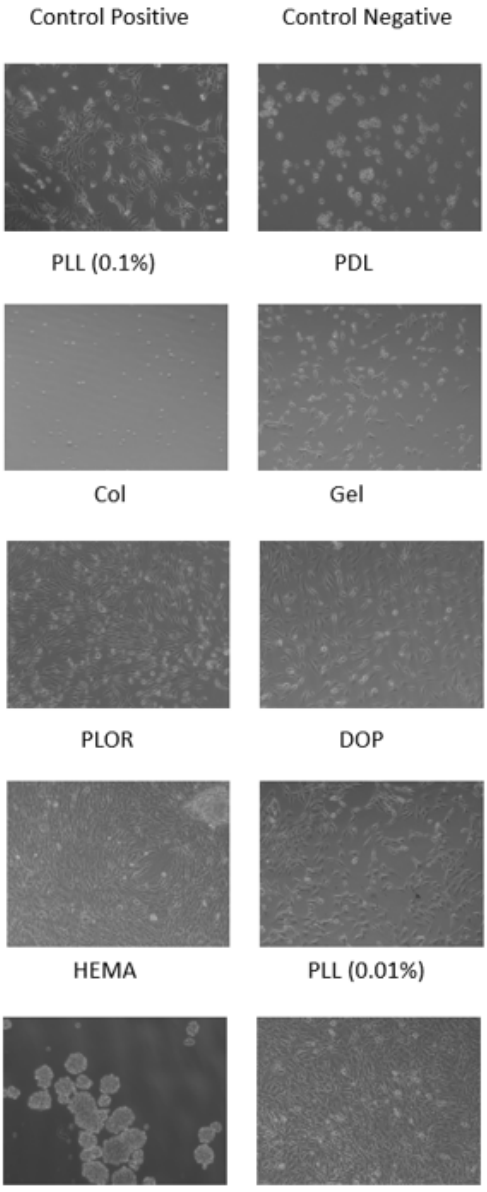

Figure 3: 3T3 Cells on Coating Materials Day 2. The figure above represents images of 3T3 cells seeded on plates 2 days prior with varying coating materials.

Coating Material	Observation
Control Positive	Highly confluent
Control Negative	Very high confluency
	Clumps of cells, no growth
Col	Highly confluent
	Highly confluent
Gel	Highly confluent
	Highly confluent
PLL (0.01%)	Highly confluent
	Highly confluent
PLL (0.1%)	Not sticking or growing
	Not sticking or growing
PDL	Sticking, not growing
	Highly confluent
PLOR	Highly confluent
	Highly confluent
DOP	Highly confluent
	Highly confluent
HEMA	Clumps of cells, not sticking
	Clumps of cells, not sticking

Table 2: Coating Observations Day 7. The table above represents the observations the team made for each of the coating materials on the seventh day after initial seeding.

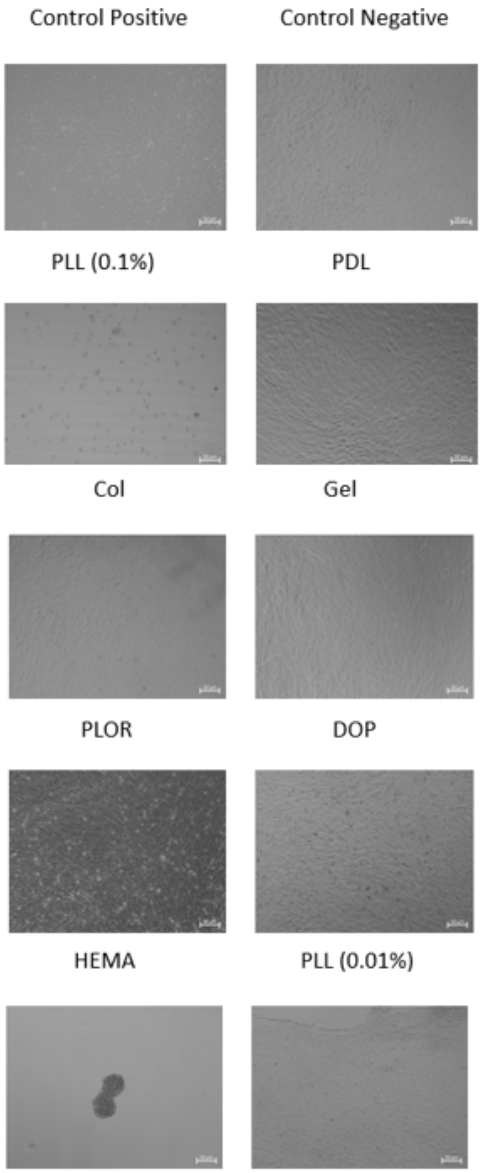

Figure 4: 3T3 Cells on Coating Materials Day 7. The figure above represents images of 3T3 cells seeded on plates 7 days prior with varying coating materials.

D. Biocompatibility

The team imaged wells of the 8 different adhesive materials 2 days after the initial seeding. With each of these images, the team made observations relating to how the cells were growing on each well. The team observed the adhesive materials 7 days after the initial seeding but did not image wells.

Coating Material	Observation
Control	Growing healthy
Acrytol	Growing healthy
Cytoseal	Not sticking, not clumped
Rubber Cement	Not sticking, beginning to clump
Silicone Glue	Not sticking, large clumps
Double-sided tape	Growing healthy
Nail polish	Not all sticking, but growing
Liquid band-aid	Not sticking, large clumps
Gorilla glue	Not sticking, no clumps

Table 3: Biocompatibility Observations Day 2. The table above represents the observations the team made for each of the adhesive materials the second day after initial seeding.

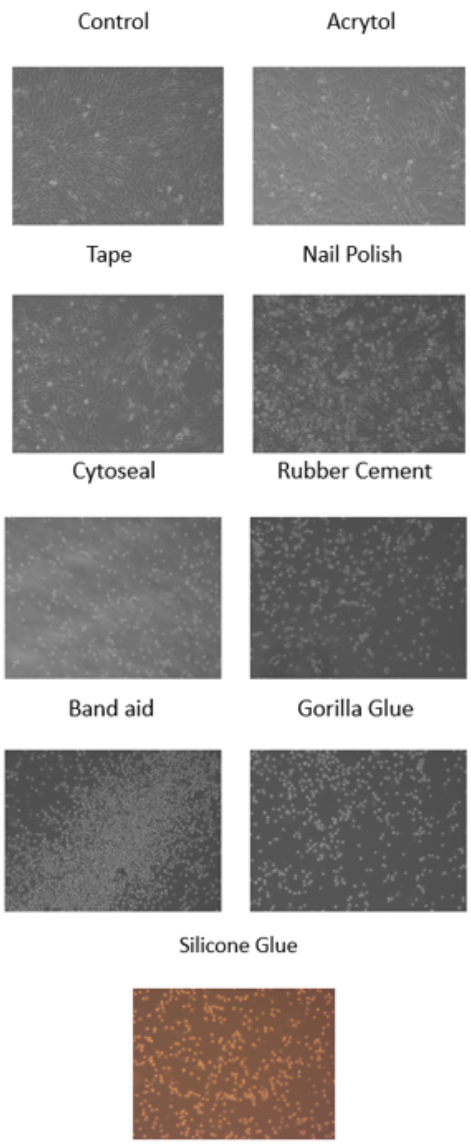

Figure 5: 3T3 Cells on Adhesive Materials Day 2. The figure above represents images of 3T3 cells seeded on plates 2 days prior with varying adhesive materials.

Coating Material	Observation
Control	Highly confluent
Acrytol	Growing healthy
Cytoseal	Stuck, not growing
Rubber Cement	Floating, not growing
Silicone Glue	Most attached, not growing
Double-sided tape	Healthy growth
Nail polish	Some growing, clumping
Liquid band-aid	No growth, large clumps
Gorilla glue	No growth, large clumps

Table 4: Biocompatibility Observations Day 7. The table above represents the observations the team made for each of the adhesive materials on the seventh day after initial seeding.

IV. Discussion

For the results of the coatings. The team originally found that the negative control, the 24 well-untreated plate was not as suitable for cell growth as one of the wells had cell confluency and the other had clumps of cells. Upon further investigation of this, the second well for the negative control had such a high confluency, the cells created a sheet and pulled off the plate which proved that the negative control was indeed a suitable source for cell growth still. This same unevenness occurred with the PDL coating as, throughout the study, one of the wells fostered health and confluent cell growth while the other did not have the same result, this cause of this could be determined to be that not enough PDL was used to coat the second well, causing abnormal cell growth.

The team mainly focused on three coating materials and expected all to be beneficial for cell growth. These materials were collagen, gelatin, and PLL. Both collagen and gelatin gave results as expected with highly confluent cells in their respective wells. The team believed that the PLL with a concentration of .01% would fare better for cell growth as compared to the .1% PLL. Upon testing both the PLL concentrations, the .01% PLL allowed for confluent cell growth while the .1% concentration left the cells unable to adhere to the plate entirely.

For the remainder of the coatings, the team was not as focused on making a hypothesis, except for the thought of the Poly-HEMA allowing for cell growth. The results of all of these coatings showed that the PLOR and DOP both allowed for confluent cell growth and against our hypothesis, Poly-HEMA caused the cells to clump together and they did not adhere to the plate.

For the positive and negative controls, the difference being a treated plate, both had a high confluency with the only difference being that one of the negative controls had cells so confluent they pulled off of the surface, besides this, both promoted very healthy cell growth.

For the biocompatibility of the adhesive material, the team believed that the biocompatible materials would be tape, liquid Band-Aid, silicon glue, Acrytol mounting media, and the cytoseal mountant. And the toxic materials would be rubber cement, nail polish, and the gorilla super glue.

After conducting the tests, for the biocompatible materials, our hypothesis of acrytol mounting media and tape were correct. The cells in the wells with these adhesives grew healthily and remained attached to the plates. Our hypothesis was incorrect for the silicone glue, liquid Band-Aid and cytoseal mountant. We believed that since liquid Band-Aid was used on the human body that it would be very biocompatible with the cells, but the cells did not adhere to the plates and did not grow whatsoever, they formed large clumps. The same hypothesis was set forth for the silicone glue as silicone is widely used in areas of human consumption such as food preparation, but it was not the most biocompatible as the cells mostly adhered but did not grow. The cytoseal yielded results similar to that of the silicone glue.

For those that we hypothesized as toxic, we were correct for rubber cement and the super glue. The super glue yielded cells that were not growing nor adhering and the cells mainly clumped up. For the rubber cement, no cells adhered to the plate and therefore did not grow. The nail polish, on the other hand, yielded some growing cells but at a very slow rate. This came as a surprise as we believed that the nail polish would be completely toxic as it is used superficially on humans and not near sensitive areas, only the nails.

In terms of adhesion, we hypothesized that the nail polish and the tape would not last when the PDMS is submerged in the complete media, but to our surprise, all of the adhesives lasted the seven days of testing but with varying levels of clarity for imaging.

V. Conclusion

Overall, with the plethora of materials used, many of our hypotheses were correct but there were quite a few surprises in terms of biocompatibility. Using the data gathered we are moving forward with the coating materials: Gelatin, collagen, PDL, and PLOR as they yielded favorable results. And for adhesives, the acrytol mounting media will be used as it was very biocompatible, yielded clear imaging, and had some bubbles over time, but the advantages that it set forth outweighed the disadvantages, and the coating will only be applied to the edges of the PDMS to allow for clear imaging.

VI. References

[1] Fox, S. (2016). *Human Physiology* (14th ed.). McGraw-Hill Education.