Lab Report #2

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***Abstract*— 3T3 cells, also known as mouse fibroblasts, were used to test the effects of a cryoprotective agent, Dimethyl Sulphoxide. The team prepared freezing mixtures and froze the cells in media with or without dimethyl sulphoxide. Three samples used dimethyl sulphoxide while only one sample was prepared without it. Once these mixtures were prepared, they were placed in the freezer at -20°C; then they were transferred to -80°C freezers. They stayed there for one week. After one week, the team thawed the cells, took an image of them, and then placed it in the incubator. After one day, the team revisited the cells and took another image. The team found that without dimethyl sulphoxide in the freezing mixture, the cells wouldn't survive, proving the importance of a cryoprotective agent. 3T3 cells were also tested to see the effects of phalloidin on actin cytoskeleton. The cells were fixed, using formaldehyde, permeabilized, using 0.1% Triton X-100, exposed to BSA blocking solution, then AlexaFluor 488-Phalloidin reagent was added. When using a fluorescent microscope, it is clear that phalloidin increases the visibility of individual fibers using different colors; there is a clear distinction between the nucleus and fibers. Phalloidin is a useful tool when needing to observe and analyze the actin of cells.**

I. Introduction

Cryopreservation of cells offers many benefits in the laboratory. In general, freezing allows for the storage of biological materials while also putting a pause on biological activities. As long as the temperatures are as low as -196 °C, it provides indefinite storage of the cells without deterioration and minimizes the chances of contamination. Primary cells are known for having a finite lifetime. With the help of cryopreservation, those cells can be preserved, preventing senescence, and allowing for consistent experimentation. Cell lines are known for having an infinite lifespan. The benefits of cryopreservation of cell lines are that it minimizes genetic mutations and variants, also allowing for a consistent experiment. Freezing cells also allows for laboratories to share and distribute cells to others, saves reagents and supplies, and protects equipment failure (referring mostly to incubators). When freezing cells, it is important to remember proper handling and harvesting of cultures, correct use of a cryoprotective agent, controlled rate of freezing, and proper cryogenic conditions to store the cells. For example, when freezing cells, it is important to use cryovials and not regular centrifuge tubes. Thawing of cells requires as much thought as well. If cells are thawed too slowly or too quickly, it can cause shock to the cells. Placing the cryovials (not fully immersed) in lukewarm water allows for a steady rate to thaw, preventing shock.

Immunocytochemistry is a laboratory technique that is used to locate and highlight parts of cells by using antibodies to bind to them. This technique can be used for a variety of reasons, one of which is to help diagnose diseases like cancer. When doing this technique, there are many steps and principles to follow. First, the cells must be seeded on the plate or well. Then the cells are fixed, using a fixative like formaldehyde, so that it may be ready to make the cell membrane permeable, using chemicals like 0.1% Triton X-100. Afterward, a blocking buffer should be added to prevent non-specific binding; one example is BSA blocking solution. That's when the antibodies or the proteins are finally added to the cells so that they can bind to the part that is going to be studied. Using these techniques helps to analyze specific parts of the cell. For example, using a purified toxin called phalloidin conjugated with Alexafluor-488 green fluorescent protein can bind to actin. Actin is a form of microfilament in the cytoskeleton; it is a protein that is important in contracting muscle cells. Phalloidin is used because it has a high affinity for actin cytoskeletal proteins.

II. Materials and Methods

A. Freezing Mammalian Cells

In this experiment, the team prepared freezing mixtures and froze 3T3 cells in media with and without the cryoprotectant DMSO (VWR, 45001-118). Each member of the team prepared one cryovial with DMSO and compared the three samples with one cryovial without DMSO. On each vial, team members wrote the cell type (3T3), whether or not the cryoprotectant was involved, a cell count based on that day's yield, the day's date, and the team members initials. The team also wrote their initials on the top of their own vials. Each team member produces their own freezing solutions A and B in separate 15ml centrifuge tubes (CellTreat, 229412). The freezing solution A contained two times the amount of cells

the team wanted to thaw and complete media. Each team member suspended 250,000 cells in their freezing solution A. Freezing solution B contained 20% DMSO (1 ml) and 4ml of complete media. Each team member took 0.5 ml from each sample into a CryoELITE Cryogenic Vial (Fisher, 02-912-728) and mixed the solutions with a micropipette. Each vial was transferred to the COOLCELL freezing container (VWR, 95059-860) in the -20℃ freezer. The container allows the vials to freeze at a constant rate of -1℃ per minute. After the lab, the professor moved the freezing container into an -80 degree freezer for a week.

B. Thawing and Plating Cells

First each team member filled a 15ml conical tube with 4 ml of fresh 10% complete media and placed it aside in the biological safety cabinet. The team placed the cryovials from the freezer into a small beaker with lukewarm water. The vials stood upright, but not fully immersed in water as the cells began a 2 minute thawing process. The cryovials being thawed were from the previous week in addition to one cryovial which was frozen without the DMSO cryoprotectant. Afterwards, the team dried the cryovials and transferred them to the hood. Each member used a micropipette to move the thawed contents to the 15 ml conical tube that was prepared earlier. In order to guarantee the contents of the cryovial fully thawed, the team rinsed them out using the medium from the same 15 ml conical tube. After rinsing, the team inverted the tube a few times and spun it at 206 rcf for 7 minutes. In order to remove the DMSO, team members aspirated the supernatant after using the centrifuge. Each team member resuspended their cell pellet in 5ml of complete media and seeded the solution in a 60mm cell culture plate. The team imaged the cells the day of and one day after thawing.

C. Phalloidin Staining for Actin Cytoskeleton

Each team has a 4-well plate (VWR, 62407-068) with each well containing 25,000 NIH3T3 cells (ATCC, CRL-1658). The team dumped the media into a separate container, added 500 µl of DPBS (+) (VWR, 45000-430) into each well, and then dumped the contents out again. The team repeated this one more time, added 500 µl of 4% methanol-free formaldehyde (Polysciences, 04018-1) into each well, incubated the plates for 10 minutes at room temperature, and aspirated the formaldehyde into a formaldehyde collection container. The team rinsed the wells with 500 µl of DPBS (+) twice like before continuing to remove the solution into the formaldehyde collection container. Then, the team permeabilized the cells by adding 500 µl of 0.1% Triton X-100 (VWR, EM-TX1568) per well and incubated the plate at room temperature for 10 minutes. The team repeated the rinsing process, added 500 µl of 1% BSA blocking solution to each well, and incubated the plate for 10 minutes at room temperature. The team dumped the BSA solution out of the wells into the original container and added 200 µl of AlexaFluor 488-Phalloidin (Invitrogen, A12379) reagent to two wells. The team kept the plate in a drawer as it incubated at room temperature for 20 minutes without being exposed to light. This process prevented photo-bleaching. The team quickly repeated the rinsing process for all plates while exposed to light, added 200 ng/ml DAPI stain (VWR 89139-118) to the two wells with Phalloidin reagent, and incubated the plate at room temperature for 10 minutes. Finally, the team added 500 µl of DPBS (+) and imaged the cells using filters for DAPI and Alexafluor with phase contrast and fluorescence microscopy.

III. Results

A. Freezing and Thawing Mammalian Cells

All members of the team froze and thawed their cells in their own cryovials in addition to being supplied with a cryovial which was frozen without the cryoprotectant DMSO. The team took pictures of the thawed cells both the day of and the day after the thawing procedure.

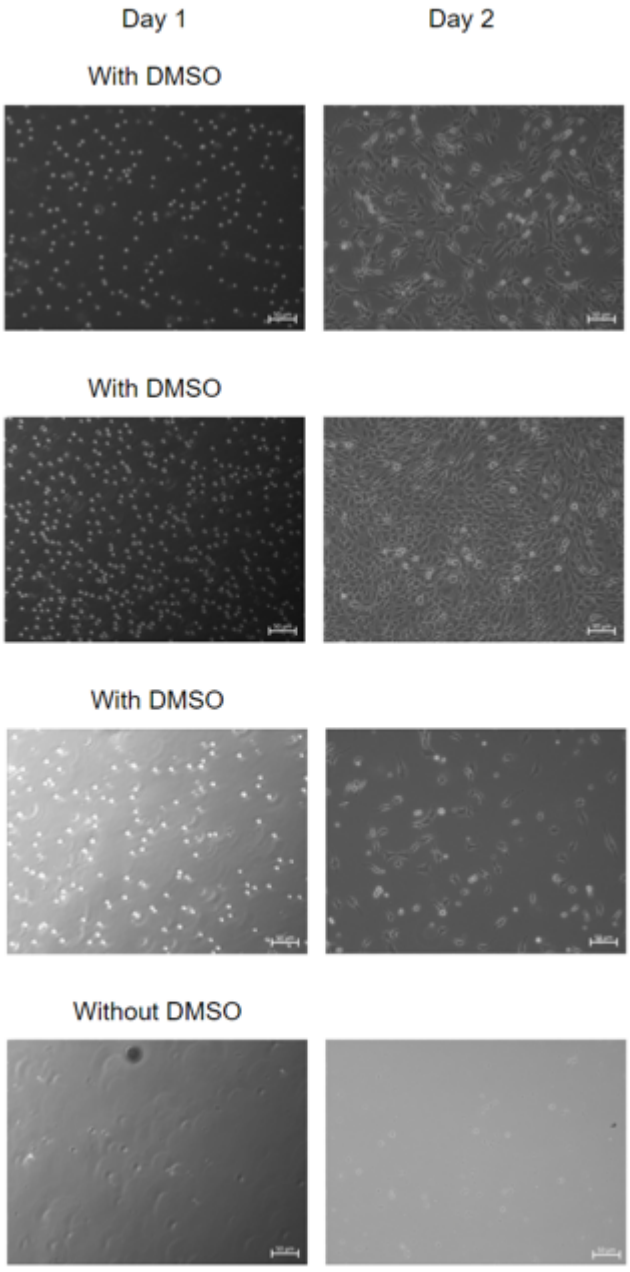

Figure 1: Thawed 3T3 Cells. The figure above represents images taken the day of and day after 3T3 cells thawed with and without DMSO cryoprotectant.

B. Phalloidin Staining for Actin Cytoskeleton

The professor took images of the 3T3 cells stained with DAPI (blue) and Alexafluor 488 (green).

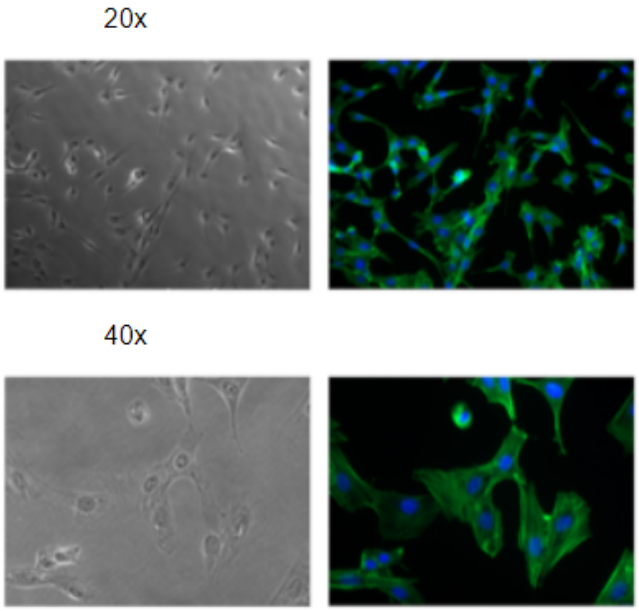

Figure 2: Stained 3T3 Cells. The figure above represents two pictures taken of stained 3T3 cells with DAPI and Alexafluor 488 at both 20X and 40X magnification.

IV. Discussion

For the thawing of cells, the cells that had DMSO in the solution fared far better than the cells that were frozen without DMSO. in comparison between the two batches of cells, less than 10% of the cells without DMSO survived, which was as expected within this lab. Although there was also variation between the three vials with DMSO can be quite large, there could be minor differences in the cells originally frozen, or where the pictures were taken relative to the location of cells.

For the staining of the Actin cytoskeleton, the distinction between the nucleus and the fibers of the cell itself is very clear, and using different colors much more can be observed. What is very surprising was the visibility of individual fibers from where cells were previously adhered that were washed off, leaving single fibers in some places that still were dyed. This ability to observe cells under the fluorescent microscope made cell imaging much clearer than the other microscopes used previously in the lab.

V. Conclusion

In Conclusion, through these experiments, we have learned about various other processes that are done with cell cultures and the materials and methods that coincide with them. For freezing and thawing, we have learned the proper ways to store cell cultures for long periods of time and how to retrieve them from long time storage. And for the staining and imaging, we have learned much more advanced imaging

techniques that are used with more advanced microscopes. With this imaging technique, we were able to see much more of the cell itself, distinguish between cells easier, and observe where cells adhere to surfaces, much to the surprise that these are all advanced capabilities that are not feasible with the normal microscopes used within the lab.

VI. References

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