Bio254 Home

Using Nile Blue Staining Techniques to Determine the Effects of Ethanol (EtOH) upon the Necrotic Cells Developing Chick Embryo Limbs

Kirsten Larsen

Web Posted on December 9, 2004

Introduction	Materials and Methods	Results	Discussion	References

To Visit Wheaton College, click [here]

I. Introduction

Fetal alcohol syndrome (FAS) is a condition caused by exposure to alcohol during embryonic development and is the leading known cause of mental retardation, producing irreversible physical and mental damage (Shea et. al, 1999). Alcohol has been shown to be a teratogenic agent, producing non-heritable birth defects, related to a mother's alcohol consumption during her pregnancy terms. Children born with FAS are usually characterized from smaller heads, a low body weight, deformed facial features and are shorter than normal. FAS can also lead to physical disabilities, problems with learning, coordination, and behavior as well (CDC, 2004). FAS is estimated to occur in 1 to 2 live births per every 1,000 in the United States each year (Abel, 1983). The effects of alcohol on developing embryos have been understood by studying embryos of other animals such as the chicken or rat. By the use of animals as models for studying the teratogenic effects alcohol in detail, the knowledge gained from these studies can be helpful in understanding the birth defects associated with this prenatal alcohol exposure.

Alcohol, along with many other toxic substances, is known to greatly affect different cells in the developing embryo, given time of exposure and susceptibility variables. As an example, the face, limbs and central nervous system all use the same set of genes to regulate growth and development, in which certain toxic substances can disrupt tissue formation (Smith, 1997). The sculpting and shaping of developing organs such as the limbs, arrive from the process of programmed cell death. Necrosis and apoptosis are two different kinds of cell death that can occur in this developmental process. Apoptosis depends on the activation of the cell's internal cell-death machinery, while necrosis occurs when cell metabolism has ceased, leading to membrane disintegration and rupturing of the cell (Smith, 1997). Through these programmed cell deaths, we get the shaping of fingers, toes and other organs. Many studies have been done on alcohol and its effects on programmed cell death in many of the animal model's embryos. By targeting the different organs of the body, these studies have provided data to support that the type of cell death working in alcohol exposed embryos is apoptotic in nature (Smith, 1997).

However in this experiment, the purpose of the study was to determine whether prenatal alcohol exposure to chick embryos had an effect on the necrotic cell death of limb development, particularly the wing. Ethanol was introduced to developing chick embryos and a process of staining the embryo itself with Nile Blue was used to determine the location of the necrotic cells of the chick wing.

It was hypothesized that if the Nile Blue staining methods worked, the developing embryos exposed to ethanol would show significantly more necrotic cells and abnormal limb bud (specifically wing) growth as compared to the control embryos. From our results, the stated hypothesis of necrotic cells was not tested, while our hypothesis about Nile Blue

methods was supported.

II. Materials and Method

Egg incubation and preparation

Eight chick eggs were placed in a 37 Celsius incubator and dated the day incubation began. On the second day of incubation, embryonic day 2, the chick eggs
were removed from the 37 C incubator and prepared for explantation of the yolk contents. A solution of 70% Ethanol was used to sterilize our hands, lab benches, forceps, weigh boats with the four corners bent upwards, paper dishes and bowls, and the chick eggs themselves and let to air dry.

Explantation of Embryos

After everything that was to be used or in contact with any tools being used was sterilized with 70% Ethanol, then explantation could begin. Upon explanting, the chick eggs were held over the paper bowl, broader side up. This broader end is where there is airspace in the yolk, so forceps were used to gently poke a small hole in this end. Upon making a hole, forceps were then used to peel away the egg shell to create a larger hole expanding the width of the airspace found. Rough corners were made smooth and a tiny hole was poked through the membrane of the yolk. After this, the egg was then flipped upside-down so the narrower end was now facing up and moved so the weigh boat was now underneath the egg. Using the forceps again, another small hole was poked at the top of this narrow end, enabling the contents of the egg insides to be released into the weigh boat beneath it. The egg shell was discarded.

Upon explanting contents into weigh boat, 0.5mL of Streptomycin (Penstrep) solution in a final concentration of 100un/100 micrograms per mL was added to all explanted embryos. This Penstrep solution is an antibiotic used to protect the chick embryos against bacteria or other infections that could alter their health. 0.5mL of 2% Ethanol solution was added into the experimental dishes, while 0.5mL of Tyrodes solution was injected into the control contents. Controls and experimentals were divided up equally among surviving explanted embryos and labeled. The explanted embryos in their respective weigh boats were then placed in a 37 C incubator.

Observing embryos

Embryos were observed daily until day 5 when they were observed finally before taken to be stained. Observations included using the Stereoscope digital interface camera and the Macintosh computers found in the ICUC lab. This camera is manufactured by Nikon and is specifically the model DAGE-MTI DC200 and it was used to take digital images with a video size of 1034 x 779 and a 4x magnification under the program of BTV Pro. This program can be found on the active desktop of all computers in the ICUC lab. Measurements of heart rate, body length, vascular area, limb bud, and wing bud growth were also taken. Heart rate was observed using a stop watch or second hand watch, in beats per minute, while the other observations were made using the mm measurements on a small ruler, although this seems like a fairly inaccurate measurement.

Making of Nile Blue staining solution (as done by Carlson and Lee, 2004.)

A stock solution of Nile Blue in HBS was made from a 1:1000 dilution by first saturating using distilled water. 20mL of distilled water was placed into a plastic 50mL centrifuge tube with screw cap. Nile Blue powder was added in small amounts until the solution was a persistent dark blue appearance. The cap was then screwed on tight and the contents were shaken vigorously for a few seconds to mix. In order to saturate the solution, more Nile Blue powder was added to the centrifuge tube until a heavy dark color persisted. This solution was assumed to be fully saturated when small clumps were visible on the side of the centrifuge tube after being shaken. Once this saturated solution of Nile Blue in HBS was made, it was used to make a 1:1000 dilution of Nile Blue in HBSS solution. This completed solution was then used to stain the chick embryos during the experimental trials performed. To make 20mL of the stain solution to be used, a plastic centrifuge tube with screw cap was filled with 20mL HBSS. 20 microliters of the saturated Nile Blue solution in distilled water was added to the 20mL of HBSS using a pipetteman. The tube was shaken for a few seconds to mix, and then immediately used. According to Carlson and Lee (2004), the remaining solutions were stored in the refrigerator for later use when not immediately used.

Nile Blue staining technique (as referred to in Grey, 1982.)
Embryos were removed from 37 C incubator and taken into the ICUC lab where a bench with forceps, paper plates and bowls, scissors, small Petri dishes and filter paper were sterilized as described above and ready for use. In order to remove the embryo from the yolk, a filter paper ring was cut and used to place over the embryo, leaving it in the center of the ring. Using a pair of forceps, the filter paper ring was lifted off, taking the embryo with it. Just before placing it in a small Petri dish containing a solution of Nile Blue and Tyrodes, where it was kept for 15-30 minutes, the amnion surrounding the embryo itself was removed carefully using forceps to peal away this membrane. A stock solution of Nile Blue solution that was made from a 1:1000 dilution as described above was put in to a small Petri dish where the embryo was placed. The embryo was then taken out using forceps and placed in another small Petri dish of Tyrodes solution for 15-30 minutes to rinse off the Nile Blue stain to reveal necrotic cells. The embryo was then observed using the stereoscope digital interface camera and methods described above to determine whether the staining technique worked and to make observations of the necrotic cells on the wing buds.

Two trials of these above methods were run, each having eight chick eggs to begin with, and depending on the survival of these embryos, controls and experimentals were made. Control chick embryos contained an injection of Tyrodes solution and were necessary to observe the normal developmental process of chick embryos in order to compare to the experimental embryos containing 2% Ethanol injections.

Statistical and Quantitative Analysis
To determine whether the Nile Blue staining worked on the embryos, images of the stained embryos were taken using the stereoscope camera and methods described previously. The image of the control embryo was taken, saved and opened using the Adobe Photoshop program found on the active desktop toolbar of the Macintosh computers. The photo was examined using a tool to select four small sections of the wing bud (visually blue section) and of the embryo body (visually non-blue section). Analysis further continued by opening the histogram tool from the Images headline in Adobe Photoshop. Histograms of three channels red, green and blue, were established for each of the four sections selected for in both the wing and embryo body of the control chick. The resulting means of each of the three channel colors from both the blue and non-blue sections given by the histogram analysis tool were averaged according to their color. Using these means, proportions between blue vs. green and blue vs. red color channels were calculated by dividing the average blue mean by the average green or red mean. This was done for both the blue and non-blue sections analyzed. This data was then put into graphical form using the program Microsoft Excel. Linear graphs were made to depict the measured means of visual blue and non-blue sections taken from the control chick embryo.

III. Results

Nile Blue staining
Through a total of two trials and sixteen chick eggs originally set aside for our use, there were only two surviving embryos that lived long enough to stain with Nile Blue. These two embryos were a result of the second trial, in the first trial there were no survivors. One embryo was designated as the control while the other was the experimental. As a result of staining these two embryos using Nile Blue technique it was shown that the embryos were stained blue, proving that this type of technique worked. As shown in Figure 1, both the control and experimental chick embryos are visually tinted blue, darker in some areas than others. This figure is used to demonstrate the resulting colors of the Nile Blue stain in the control and experimental chick embryos. It is also used to show the locations of the sections of the control chick embryo's wing and body that were statistically analyzed. Channel settings of the colors red, green and blue were used to determine the mean for each color absorbed. In examining the sections of the control embryo's body, which was visually non-blue, the mean for each of these three color channels showed to be roughly the same in comparison. As shown in Figure 2 as an example of the histogram analysis to be taken at all four sections of the visually non-blue section, the means of the control embryo ranged from 233.43 to 254.20. This shows that all colors are being absorbed at about equal amounts and the result would be a non-blue stain as indicated in Figure 1 B. represented by the lesser tinted areas on the embryo. Furthermore, Figure 4 depicts the total means and variations of the three different color channels observed in the embryo's visually non-blue section in graphical form. Also, in using

the average means taken from each of the three color channels, proportions of blue vs. green, and blue vs. red were interpreted. The average means were as follows: Blue 254.20, Green 253.18, Red 232.93; showing a relatively close comparison of the average means between the three color channels. Proportion analysis showed that when comparing the blue to green channel, there was a proportion of 1.0. When comparing the blue to red channel, there was a proportion of 1.1. These proportions further show how closely related the three colors are to one another, being thought of as a 1:1 ratio, suggesting that these channels of color are being absorbed equally, reflecting a white, non-blue color that is visually seen.

In examining the wing sections of the control embryo which was visually blue in color, the means for the three color channels were also compared as mentioned above. Here, the average means were as follows: Blue 221.83, Green 155.69, Red 126.31; with the blue channel showing a marked difference in the average mean as compared to the green and red channels. Figure 3 is used as an example of the histogram taken from a section of the wing in the control chick embryo to show the variation of the means of these three color channels. Additionally, Figure 5 depicts the total means and variations of the three different color channels observed in the embryo's visually blue section in graphical form. In performing proportion analysis between the blue and green channels, there was a proportion of 1.5; while the blue and red channels yielded a proportion of 1.8. These higher proportions are an indication of a higher ratio between blue to green and blue to red: suggesting greater amounts of blue light are being reflected off the surface of the embryo so that the color blue is visually seen.

Necrotic Cell pattern

The results gathered from the Nile Blue staining were not sufficient enough to produce valid conclusions regarding necrotic cell patterns, locations or abnormal growths. Although the Nile Blue was successful in staining the embryo, our methods of doing so were unsuccessful. Upon placing the embryos into the Nile Blue stain, the amnion of both embryos failed to be removed, therefore only staining this portion of the embryo. The amnion had to be taken off using forceps, and the embryo then placed back into the Nile Blue solution to be re-dyed. Also, each of these Petri dishes containing Nile Blue and Tyrodes solution was supposed to be warm to keep the cells alive for a longer period of time and allowing the necrotic cells to be easily determined. However, the Petri dishes were also not kept warm, therefore influencing the cells that were once alive to become necrotic and interrupt the observations trying to be made. As a result of these methods, the necrotic cells were unable to even be seen, even though the Nile Blue stain produced results. There were also no visible differences or abnormalities in limb development between the two embryos observed.

Figure 1 (below): Visual appearance of blue tinting over both the experimental (top) and the control (bottom) chick embryos are shown here as a result of the Nile Blue staining. Statistical analysis was done on four sections of the control embryo's wing and control body in order to obtain results mentioned.

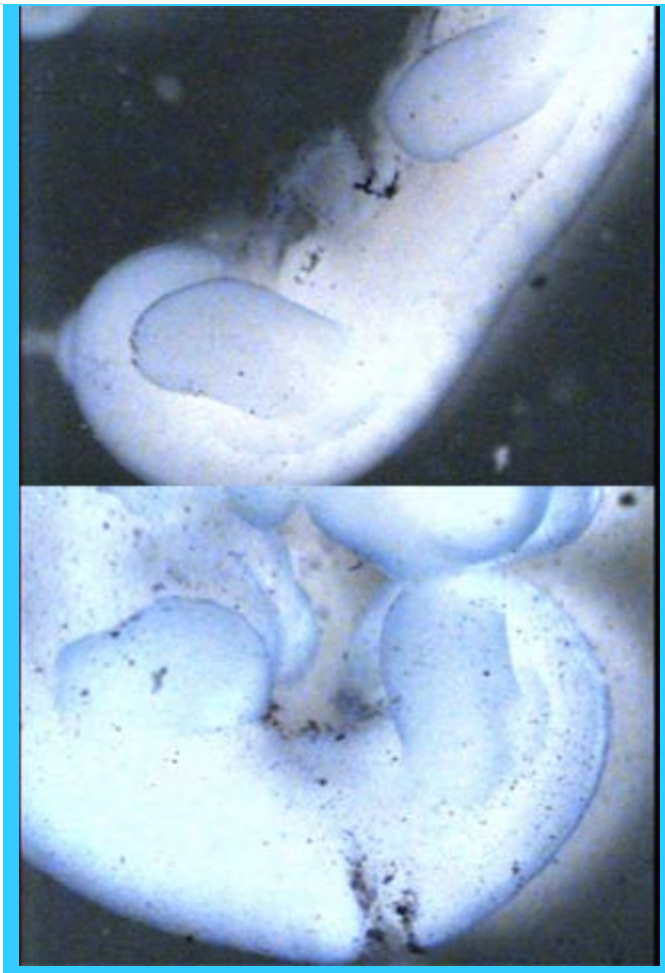


Figure 2 (below): Histograms of three colors as a result of Nile Blue staining. This is an example of the data that was collected in a non-blue wing region from the control embryo.

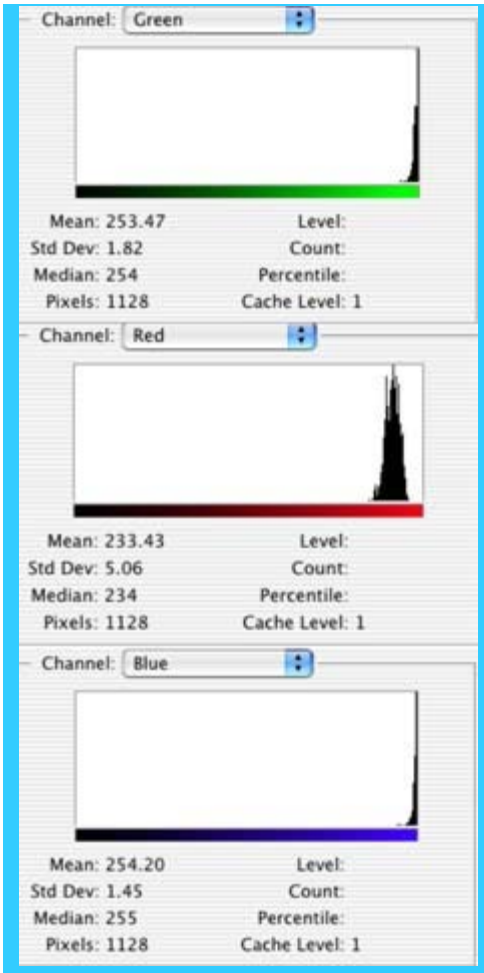


Figure 3 (below): Histograms of three colors as a result of Nile Blue staining. This is an example of the data that was collected in a blue wing region from the control embryo.

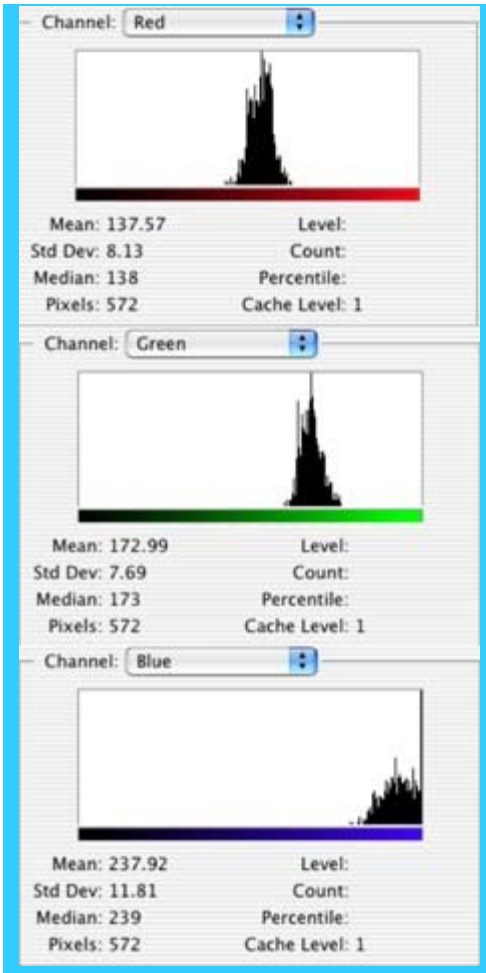


Figure 4 (below): Graph depicting the total means and variations of the three different color channels observed in the control embryo's visually non-blue sections of the body.

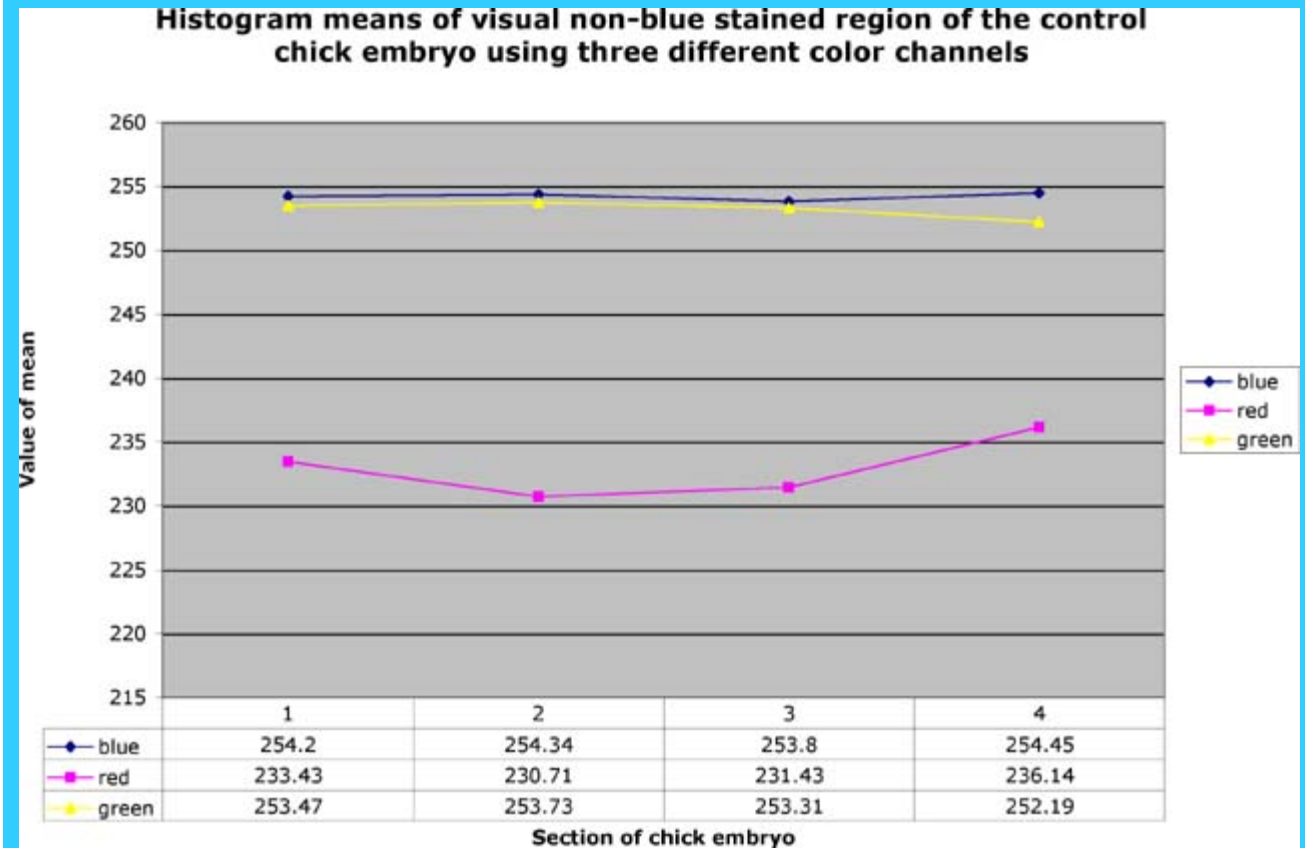
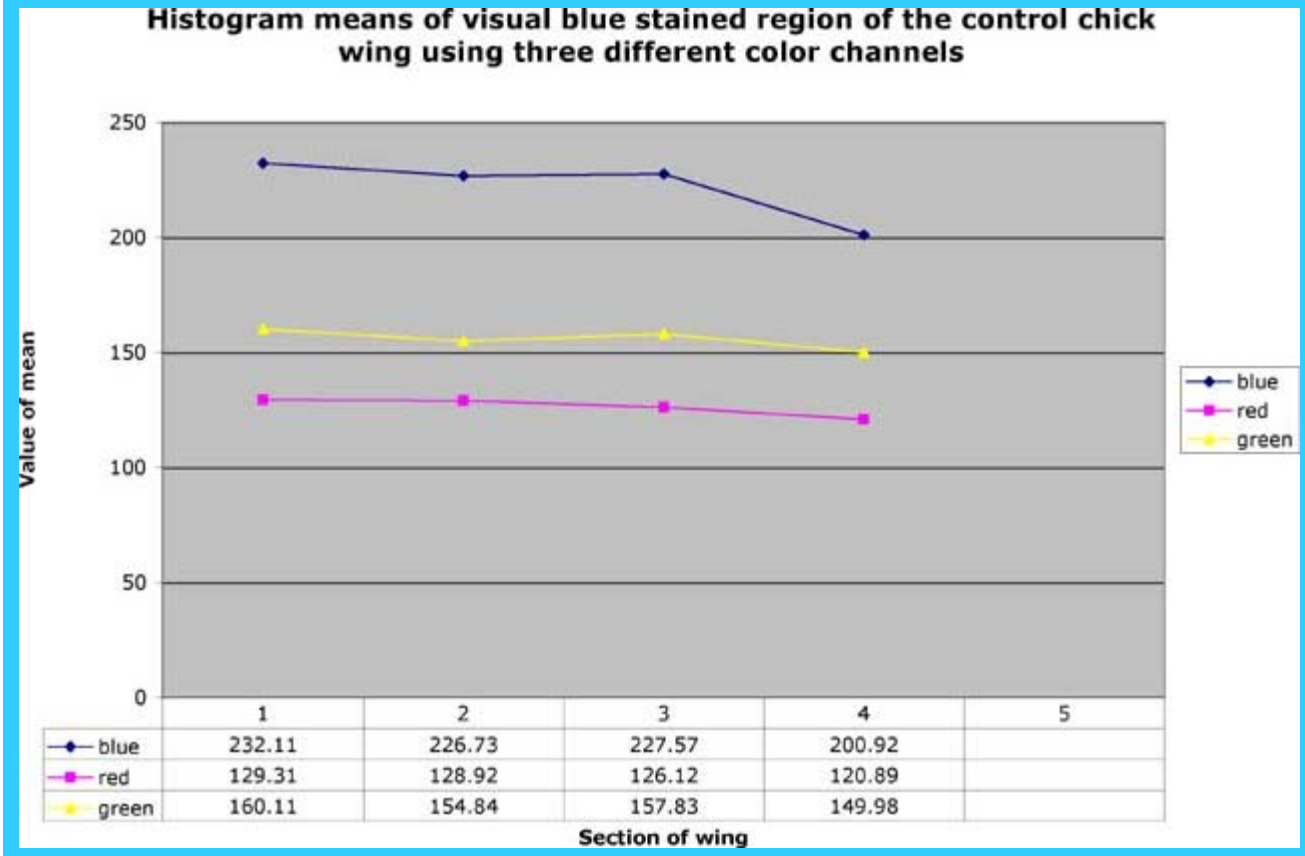


Figure 5 (below): Graph depicting the total means and variations of the three different color channels observed in the control embryo's visually blue sections of the wing bud.



	1	2	3	4	5
blue	232.11	226.73	227.57	200.92	
red	129.31	128.92	126.12	120.89	
green	160.11	154.84	157.83	149.98	

IV. Discussion and Conclusions

Nile Blue staining technique

We have been able to produce a Nile Blue staining technique that shown to stain the chick embryos blue in color. Histograms of two different visually colored areas in the control chick embryo were examined by comparing three color channels, red, green and blue, and their respective means. As a result of the statistical analyses, we were able to demonstrate that the Nile Blue staining technique began to provide us with a darker blue stain in the areas where there may have been more concentrated necrotic cells on the wing buds, as opposed to the visually non-blue areas of the embryo's body. This result supported one of our initial hypotheses.

Necrotic Cell evaluation

Although this technique of Nile Blue staining worked, we were unable to evaluate the necrotic cells in the limb buds of the chick embryo, proving whether there were greater numbers of necrotic cells or any areas of abnormal growth of the limbs, particularly the wing, themselves. This result rejected one of our initial hypotheses due to many sources of error that occurred during this experiment.

Sources of error in this experiment

There were many sources of error that occurred during our experiment that greatly affected the data that we hoped to collect. To begin with, there were no surviving chicks in our first trial round. We began with only five chick eggs at the start, three were stolen. Upon explantation of these five, the yolks either broke or the embryo itself showed no signs

of life. Trial one therefore yielded no results and we were unable to perform our Nile Blue staining. If this trial produced live chicks, we would have had been able to test our staining methods and further improve on them during our second trial. Our second trial therefore was our first for the staining technique that we hoped would work properly enough to obtain necrotic cell results. In this second trial, we began with eight chick eggs, and through explantation we only had two surviving chick embryos, one that we designated the control, the other the experimental. Although this was significantly better than our results from the first trial, it would have been nice to have all eight embryos alive to make greater comparisons between the controls and experimental embryos.

Upon the staining of these two surviving embryos, we failed to remove the amnion surrounding the embryo on both the control and experimental. We realized this after we let the embryo sit in the Nile Blue stain for roughly 15 minutes, after which we took the embryos out, removed the amnion and replaced them back into the Nile Blue solution. The transferring and staining multiple times of the embryos could have greatly affected the status of the embryo, as well as the amount of necrotic cells, which could have easily increased due to these unstable conditions the embryo was put through.

The procedure that we were following required that the solutions of Tyrodes and Nile Blue be kept warm in order to ensure that no new necrotic or dead cells appeared while the staining was taking place. However, we did not warm these solutions, rather used them at room temperature and/or slightly refrigerated. These temperatures at which we exposed the embryos to could have also affected the number of necrotic cells. The purpose of warming the solutions was to ensure that the cells that were still alive and well kept alive and well, instead of leading to more necrotic cells, disrupting our data that we hoped to obtain.

Upon explantation of the embryos, especially in the first trial that was ran, we could have been even more careful with our techniques used. We could have also tried another method of explanting, the cooks method, in which the egg would be cracked gently on the side of a countertop or using forceps and the contents be exposed as though a cook would be cracking an egg into a frying pan. This method could have produced greater results in the survivability of our embryos.

Future experiments

There have been many studies done on producing fate maps of the chick limb bud by scientists and researchers. Such fate maps have been produced in detail to show how the wing or leg bud arises in development using methods of DiI microinjections and other cell staining techniques. As a result of these experiments, researchers such as Vargesson et al. have been able to further understand the development of the chick limbs under normal conditions (Vargesson et al, 1997). Experiments such as the Nile Blue staining of necrotic cells that we tried to perform can be done to develop fate maps of embryos exposed to doses of ethanol, lead acetate or other chemicals. These fate maps can be done to further understand the methods to which these toxins act on developing embryos in animals and hopefully be used to understand human development as well. If our experiment was done properly and with perhaps greater technology available to us, we could reproduce such fate maps of necrotic cells in the limbs of chick embryos to understand the basis of fetal alcohol syndrome upon limb development in chicks and hopefully compare the results to that of human fetal alcohol syndrome.

Special thanks to my lab partner Molly Troup who endured many long days and nights in the ICUC lab with me. Also to my classmates Liv Carlson and Jon Lee, without whom we would be lost with our Nile Blue solution.

V. Bibliography

Carlson, Liv and Jon Lee. 2004. Developmental Biology 254. Wheaton College. Norton, MA.

Centers for Disease Control (CDC). 2004. Fetal Alcohol Syndrome. Medline Plus. http://www.cdc.gov/ncbddd/fas/

Gordis, Enoch M.D, Commentary NIAA Director. 2004. Fetal Alcohol Syndrome. http://alcoholism.about.com/cs/alerts/1/blnaa13.htm

Grey, R.D., P.B. Armstrong, C.A. Erickson, and A.C. Lupo. 1982. A Laboratory Text for Developmental Biology; Observations of Living Embryos. Chapter 5. University of CA, Davis.

Hamburger, Victor, and Howard L. Hamilton. Stages of Chick Embryo Development, A Series of Normal Stages in the Development of the Chick Embryo. Pages 56-62

Shea, Kathryn, and Stephen Winners. Northeast Consultation and Training Center. 1999. Facts about FAS/FAE. http://www.taconic.net/seminars/fas-a.html

Smith, Susan M. Ph.D. 1997. Alcohol-Induced Cell Death in the Embryo. Vol. 21, No. 4. University of Wisconsin, Madison.

Vargesson, Neil, Jonathan D. W. Clarke, Katherine Vincent, Clare Coles, Lewis Wolpert, and Cheryll Tickle. 1997. Cell fate in the chick limb bud and relationship to gene expression.

Yokouchi, Yuji, Jyun-Ichi Sakiyama, Takashi Kameda, Hideo Iba, Atsushi Suzuki, Naoto Ueno and Atsushi Kuroiwa. 1996. BMP-2/4 mediate programmed cell death in chicken limb buds.