

# The Relationship between Methylmercury Dosages and Amount of Endocytic Activity in Primary Culture Chick Glial Cells

Dana MacDonald  
Neurobiology Short Report  
Bio 324/Neurobiology  
Wheaton College, Norton, Massachusetts, USA  
April 23, 2014

## Introduction

Cellular mechanisms serve a variety of roles within the body and can either benefit or harm the host, depending on numerous factors. One of these mechanisms is a series of steps known as endocytosis. While endocytosis takes place in cells all throughout the body, this experiment focuses on the behavior in the most abundant cells in the nervous system, glia (Freeman, 2006).

Endosomes are membrane-bound vesicles within the cytoplasm that form at the start of the endocytic pathway. The plasma membrane engulfs extracellular material by folding inward. This portion of the membrane buds off and an endosome forms inside the cell. The endosome carries its contents to a lysosome, which fuses with the membrane of the endosome and digests or breaks down its contents (Davidson, 2005). The contents of endosomes can range from valuable nutrients to harmful toxins depending on the extracellular environment, and can therefore, have potentially dramatic effects on the host (Mukherjee, Ghosh, & Maxfield, 1997). For a toxin to perform its harmful action, it must enter a cell via endocytosis (Reig & van der Goot, 2006). This experiment attempts to examine the endocytosis of methylmercury, a toxin in the environment that has widespread effects on the nervous system (Kaur, Aschner, & Syversen, 2011). A toxin's presence in the cell's environment simply makes it possible for that toxin to enter a cell and be contained in an endosome. So, the introduction of a toxin to a cell's environment leads to a likelihood of toxic endosomes forming inside.

Methylmercury is a particularly important toxin to further understand because of its bioavailability in conjunction with its potentially devastating adverse effects on the nervous system. Bacteria methylate mercury in aquatic environments (Davidson et al., 1998). Bioaccumulation and biomagnification then occur up the aquatic food chain. The main source of this neurotoxin in humans is through fish consumption (Lebel et al., 1997). While fish are a nutritious source of protein and omega-3 fatty acids that benefit neurological development, these benefits are thought to be reduced by the profound negative effects of methylmercury (Mergler et al., 2007). Exposure to methylmercury has been associated with a wide range of visual, motor, and cognitive deficits (Mergler et al., 2007). These deficits include the constriction of visual fields, paresthesia, ataxia, dysarthria, auditory disturbances, speech impairment, and tremor (Mergler et al., 2007; Griesbauer, 2007). These adverse effects are especially damaging in utero. Methylmercury is transferred across the placenta and to the developing fetus when a mother is exposed to the toxin. While the mother could remain unaffected, fetuses that were exposed to methylmercury may become mentally retarded (Mergler et al., 2007). High exposure to methylmercury in utero has been shown to be associated with low neonatal neurologic optimality scores (Steuerswald et al., 1999). Previous studies of the effects of methylmercury have utilized hair as a reliable biomarker in measuring methylmercury exposure and have observed these many associated neurological impairments (Mergler et al., 2007).

The impact of methylmercury specifically on glial cells is significant due to the vital role of these cells in the nervous system. Glial cells regulate many important functions of the nervous system including nerve signal propagation, neurotransmitter uptake, synaptic action, axon pathfinding, and nervous system assembly and development (Purves, Augustine, & Fitzpatrick, 2001; Freeman, 2006). This experiment studies glial cells in chicks (*Gallus gallus*) growing in vitro (R.L. Morris, personal communication, April 2014). This system is an accessible one that serves to model humans. The study aims to observe the endocytic behavior of glia with and without exposure to methylmercury. I tested the hypothesis that as the dose of methylmercury increases so does the amount of endocytic activity within glial cells. In this study, I applied a methylmercury solution to primary culture chick glial cells, labeled the endosomes using a fluorescein-dextran solution, captured images to observe the amount of endocytic activity, and compared my findings with a control condition that I created using similar protocol. By looking at the effects of methylmercury on chick glial cells, one can begin to better understand the mechanisms by which the toxin affects the nervous system. With a more complete understanding of the impact of methylmercury on glial cells as it relates to endosome abundance, a better foundation of toxin-related diseases in humans can result (Benoit, 2014).

## Materials and Methods

### Dissection

A protocol outlined by Robert L. Morris was followed to isolate the dorsal root ganglia and sympathetic nerve chains from ten day-old chick embryos (Morris, 2014a). The following modifications were made to this protocol. DMEM (Dulbecco's Modified Eagle's Medium) was used instead of HBSS (Hank's Balanced Salt Solution). The flame-constriction of Pasteur pipettes was not performed. The coverslips were treated with poly-L-lysine for three hours to increase stickiness and with laminin for two hours to increase signaling. The concentration of NGF (nerve growth factor) in the growth medium was 200 ng/ml to increase axon growth rate. The concentration of glutamine used was 4 mM due to its tendency to break down. The shelf in the incubator was isolated from vibrations to increase the adherence of the cells. The dissection was also performed 36.5 hours prior to the creation of the control and experimental conditions.

### Control Condition

To create a control condition of no methylmercury exposure, a petri dish was first obtained from the incubator and carefully transported to a lab bench. The petri dish contained a coverslip in F-plus growth medium that had cells that were plated thirty-six and a half hours prior. Once removed from incubation, the F-plus growth medium was extracted from the culture using a Pasteur pipette. DMEM was added with a Pasteur pipette in an amount necessary to cover the plated cells. The petri dish of cells was incubated for twenty minutes. Following this incubation, the DMEM was withdrawn with a pipette and the coverslip was washed once with fresh DMEM. All DMEM and F-plus medium throughout this procedure was disposed of in waste containers.

### Experimental Condition

To create an experimental condition of methylmercury exposure, a petri dish was first obtained from the incubator and carefully transported to a lab bench. The petri dish contained a coverslip in F-plus growth medium that had cells that were plated thirty-six and a half hours prior. Once removed from incubation, the F-plus growth medium was extracted from the culture using a Pasteur pipette. A solution of DMEM and methylmercury was added with a Pasteur pipette in an amount necessary to cover the plated cells. The concentration of this solution was 40 nM MeHg in DMEM. The calculation of this concentration was based on an experiment by Leong, but modified for whole culture conditions and direct application to all cells (Leong, Syed, & Lorschieder, 2001). The petri dish of cells was incubated for twenty minutes. Following this incubation, the solution of DMEM and methylmercury was withdrawn and the coverslip was washed once with fresh DMEM. The withdrawn DMEM and methylmercury solution was disposed of in an appropriate waste bottle.

### Endosome Labeling

To label the endosomes in both the control and experimental conditions, the same procedure was followed. First, the DMEM from the wash removed using a Pasteur pipette. Two milliliters of fluorescein-dextran solution was prepared with a concentration of 10  $\mu$ M in growth medium. The calculation of this concentration was based on a study by Bi and Morris (Bi et al., 1997). The solution was approximately divided in half and added to the two dishes so that each contained roughly one milliliter of fluorescent dye. Immediately after, the dishes were covered with aluminum foil to keep light from breaking down the solution. The dishes were then incubated for forty-five minutes. Using a Pasteur pipette, the fluorescein-dextran solution was removed from both dishes following incubation. The coverslips were washed with fresh DMEM three times. After the third wash, a color assay was performed in order to be sure that the washes were successful. The coverslips were washed a fourth time so that the solution on the cells was the pink color of DMEM and no traces of the yellow fluorescein-dextran could be seen. The endosome labeling procedure was based on a protocol by Robert L. Morris (Morris, 2014c).

### Observation Chamber Assembly

Following the endosome labeling procedure, an observation chamber for each of the two conditions was made using the chip chamber section of a protocol by Robert L. Morris (Morris, 2014b). The slide with the coverslip containing cells that were treated with methylmercury was labeled experimental and the slide

with the coverslip containing cells that were not treated with methylmercury was labeled control.

#### Microscopy

Once in the ICUC, the control slide was placed on a Nikon Eclipse E200 microscope stage. The microscope was aligned for Koehler illumination under transmitted light. Using the eyepieces, a bubble was brought into focus using a 10X objective. From there, the stage was dropped and the level containing cells was focused into view using the 40X objective. Next, the pin on the side of the microscope was pulled to send the image to the computer screen using the Spot Advanced program. The computer used was the Macintosh in the ICUC labeled "Gemini". After pulling the pin, the restart button was clicked to refresh the image. The image was then focused on the computer using the fine focus knob. The image was optimized on the computer screen and picture was taken. To get the same image with fluorescent microscopy, the transmitted light was first cut off. Then on the Spot Advanced program, fluorescence was selected as the image type. Manual exposure was selected, exposure time was set to ten seconds, and a gain of two was chosen. The fluorescence slider of the microscope was shifted to position three. The shutter was opened and the image was captured. The shutter was quickly closed. The transmitted light image and the fluorescent image were saved to the computer. The same process of capturing a transmitted light image and a fluorescent image was repeated twice more for the control slide and a total of three times for the experimental slide.

#### Measurement

Each of the six fluorescent images was edited in Adobe Photoshop by adjusting the brightness levels so that the fluorescence shapes within each cell could be seen more easily. The levels were adjusted the same way for each image. The transmitted light images were used to identify the boundaries of the glial cells. In the fluorescent images that were edited in Adobe Photoshop, a variety of bright green shapes were apparent, especially in the glial cell regions determined by the images under transmitted light. While it was evident that endocytic activity had been occurring in these glial cells, the exact number of endosomes could not be quantified because they were often clumped together and indistinguishable. Instead, a fluorescent spot within a glial cell region in one of the images was chosen to represent an endosome based on its shape and size. The spot was spherical like an endosome and fell within its size range of 200nm-1000nm (Duzgunes, 1993). To find out if the spot fell in this range, the line tool in ImageJ was used to determine the number of pixels per millimeter. There were 554 pixels in every 1/10 mm.

The image containing this fluorescent spot ("endosome") was opened in the application ImageJ. Under the "image" window, the 8-bit image type was selected (H. Pope, personal communication, April 2014). This selection generated an image with a black background and white spots. The white spots represented the original green fluorescence. Also under the image window, threshold adjustment was selected (H. Pope, personal communication, April 2014). The threshold images were adjusted until all white spots that were less bright than the chosen "endosome" disappeared from the image. The only white spots remaining were as bright or brighter than the "endosome". Its brightness served as the threshold. This step was done so that the fluorescence quantified to measure endocytic activity represented endosomes or clumps of endosomes and not the duller flecks resulting from noise that existed throughout each image. The five remaining fluorescent images were opened and edited in ImageJ using the same brightness threshold.

In all six of the images in ImageJ, the total number of pixels per glial cell was determined. The white shapes represented the endocytic activity by being of a certain brightness, so the number of white pixels for a given glial cell was also determined. For each of the six images, data from just one glial cell were collected. This method led to three sets of values of endocytic activity for the control cells and three for the experimental cells. Megan Donahue utilized the same method and same images to collect her data and Gaby Verde used the same transmitted light images and Adobe Photoshop images to gather data about endosome velocity.

#### Data Analysis

For each of the six glial cells from which data were collected, a ratio of white pixels per glial cell to total pixels per glial cell was calculated. To generate each ratio, the number of white pixels in given glial cell was divided by the total number of pixels in that cell. For each of the two conditions, an average ratio of white pixels to total pixels was calculated. These averages were compared to generate a conclusion about the original hypothesis.

## Results

The images of glial cells collected under transmitted light, the fluorescent images produced using Adobe Photoshop, and the brightness threshold images produced using ImageJ lead to important observations that were key in quantifying the data. In microscope images under transmitted light, I observed the boundaries and shapes of the glial cells. Figure 1 is an example of an image under transmitted light in the control condition and Figure 4 is an example of an image under transmitted light in the experimental condition. In the fluorescent images edited with Adobe Photoshop, I observed clusters of green spots in areas in the cell regions. Figure 2 is an example of a fluorescent image edited in Photoshop in the control condition and Figure 5 is an example of a fluorescent image edited in Photoshop in the experimental condition. In the black and white threshold images, white spots were contained within glial cells regions. There were visibly fewer white pixels in the ImageJ images as compared to fluorescent pixels in the Adobe Photoshop images resulting from the introduction of the brightness threshold. Figure 3 is an example of an image edited in ImageJ in the control condition and Figure 6 is an example of an image edited in ImageJ in the experimental condition.

Figures 3 and 6 represent two of the six images from which data were obtained. The data from Figure 3 were averaged with the data from two other control glial cells and the data from Figure 6 were averaged with the data from two other experimental glial cells. These two averages are displayed in a bar graph, Figure 7, in which there appears to be no difference between the two conditions.

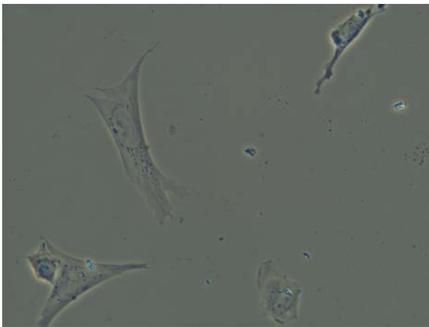


Figure 1. Glial cells in control condition under transmitted light. The glial cell in the bottom left-hand corner of the image has clearly defined boundaries that were used to determine the cell region for quantifying endocytic activity without exposure to methylmercury.

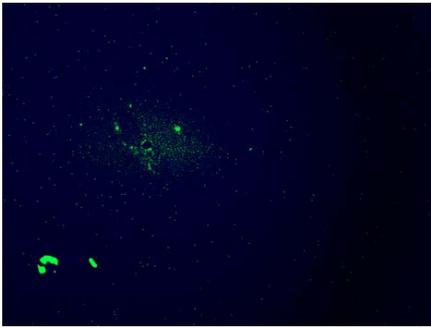


Figure 2. The image of fluorescent activity in glial cells in the control condition following editing in Adobe Photoshop. This image is the result of capturing a picture of Figure 1 under fluorescence and then adjusting brightness levels in Adobe Photoshop.

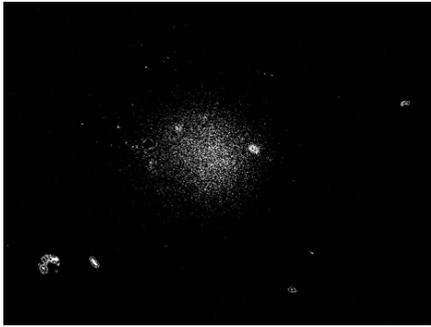


Figure 3. The brightness threshold image of endocytic activity in the control condition following editing in ImageJ. This image is the result of editing Figure 2 in ImageJ with brightness threshold. The number of total pixels and the number of white pixels in a glial cell were determined to calculate a ratio representing endocytic activity.



Figure 4. Glial cells in experimental condition under transmitted light. The glial cell on the right has clearly defined boundaries that were used to determine the cell region for quantifying endocytic activity with exposure to methylmercury.

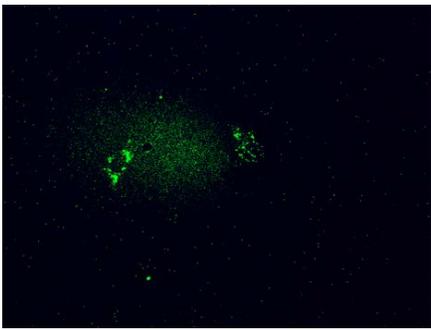


Figure 5. The image of fluorescent activity in glial cells in the experimental condition following editing in Adobe Photoshop. This image is the result of capturing a picture of Figure 4 under fluorescence and then adjusting brightness levels in Adobe Photoshop.

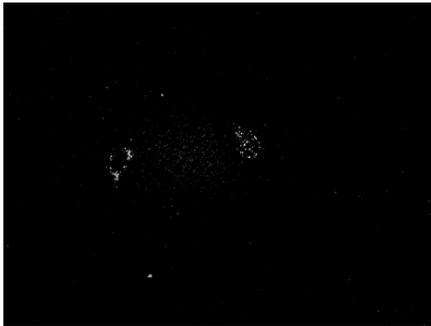


Figure 6. The brightness threshold image of endocytic activity in the experimental condition following editing in ImageJ. This image is the result of editing Figure 5 in ImageJ with brightness threshold. The number of total pixels and the number of white pixels in a glial cell were determined to calculate a ratio representing endocytic activity.

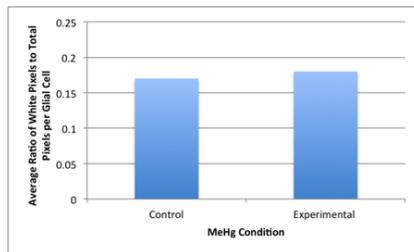


Figure 7. The average ratio of white pixels to total pixels per glial cell in the control and experimental conditions. Notice that there does not appear to be a large difference between the average ratios of white pixels to total pixels per glial cell in the control and experimental conditions (n-value= six glial cells). Megan Donahue used the same images and data collection procedure and discovered a similar trend (M. Donahue, personal communication, April 2014). Gaby Verde served as a collaborator in the collection of these data (G. Verde, personal communication, April 2014).

## Discussion and Conclusions

The hypothesis that, as methylmercury dose increases so does the endocytic activity in glial cells, is rejected by the data collected in this experiment. From these data, exposure to methylmercury does not seem to be associated with a greater amount of endocytic activity in glial cells, as compared with cells that are not exposed to methylmercury. If this experiment was repeated several times and the same results were collected from a much larger pool of data, the conclusion that exposure to methylmercury leads to more endocytosis in glial cells would be certainly not be supported. Instead, based on the small difference in the ratio of white pixels to total pixels in the two conditions, it would be concluded that there is not a relationship between methylmercury dosage and endocytic activity in glial cells. Endocytosis occurs each time that an endosome is formed by the folding inward of the plasma membrane (Davidson, 2005). Therefore, more endocytosis is the equivalent of the formation of more endosomes. Thus, if there are more endosomes in the cytoplasm of glia, then more endocytosis has taken place. If there is no difference in the amount of endocytic activity in repeated trials of methylmercury exposure to glial cells as compared to a control condition, then it can be concluded that exposure to methylmercury does not affect endocytosis in glia. This conclusion can be explained from a cellular standpoint by further considering the process of endocytosis. A toxin, like methylmercury, must gain entry into cells via endocytosis in order to perform its action (Reig & van der Goot, 2006). Cells uptake contents from their extracellular environments and so, when methylmercury is present outside the cell, it is endocytosed (Davidson, 2005). However, regardless of the amount and type of a substance present in the extracellular matrix, endocytosis occurs at the same rate (Battey, James, Greenland, & Brownlee, 1990). If other conditions are kept constant, then the presence of methylmercury does not lead to more endocytosis. Endocytosis also occurs in the cells without exposure to methylmercury. The only potential difference between the conditions is the contents of the endosomes as a result of their differing extracellular environments.

There was a particular source of error in this experiment that is worth mentioning. By understanding a weaker aspect of an experiment, it can more easily be refined in the future. While the importance of maintaining identical conditions for the experimental and control cells (excluding the independent variable of methylmercury exposure) was understood by all experimenters, there was an instance in which the two groups of cells were treated slightly differently. When capturing images in the ICUC, data from the control slide were gathered first. Data from the experimental slide were not collected until at least thirty minutes later. Therefore, time before data collection could serve as an additional variable when determining the causes of endosome abundance. To refine the experiment, this source of error should be reduced or eliminated completely if possible. Experimenters could reverse the order of the conditions during data collection so that time is no longer an additional variable. It would be a good idea for half of the experimenters to look experimental conditions first and for half to look at the control conditions and then pool their data. Also, working through the data collection more efficiently and leaving a shorter amount of time between the capturing of images in the control and experimental conditions could reduce this issue. Additionally, experimenters could look at the relationship between time and endosome abundance, without any methylmercury exposure, to determine if this source of error is actually affecting the results. If there is an observed relationship between time and endocytic activity, it is especially important that this extra variable be eliminated so that the methylmercury exposure is the only factor being studied.

There seems to be no previous work on the abundance of endosomes in glia treated with methylmercury, so more studies testing the same hypothesis should be performed. Megan Donahue used the same images to collect her data and my data are supported by her findings (M. Donahue, personal communication, April 2014). However, considering the same source of error was present and a relatively low number of glial cells were studied, it would be very informative if other experimenters took on the testing of the same hypothesis with similar methods. The topic of endocytic activity in glial cells and methylmercury exposure is an interesting one to investigate because of the bioavailability and widespread effects of methylmercury on the nervous system (Davidson et al., 1998). A future experiment should look at the effects of methylmercury on endocytic activity in neurons. Looking at endocytosis in the presence of methylmercury in glial cells only tells part of the story because neurons are also such key players in the nervous system. By studying both cells, future experiments can lead to the development of a more comprehensive understanding of the mechanisms by which methylmercury produces its adverse effects on the nervous system.

## References Cited

Battey, N., James, N., Greenland, A., Brownlee, C. (1990). Exocytosis and Endocytosis. Retrieved from <http://www.plantcell.org/content/11/4/643.full>

Bi, G. Q., Morris, R. L., Liao, G., Alderton, J. M., Scholey, J. M., & Steinhardt, R.A. (1997). Kinesin-and myosin-driven steps of vesicle recruitment for Ca<sup>2+</sup> regulated exocytosis. *The Journal of Cell Biology*, 138 (5), 999-1008. Retrieved from <http://jcb.rupress.org/content/138/5/999.long>.

Benoit J. (2014, February 13). *A Crash Course on Mercury*.

Davidson, M. W. (2005). Endosomes and endocytosis. *Molecular Expressions*. Retrieved from <http://mirco.magnet.fsu.edu/cells/endosomes/endosomes.html>.

Davidson, P.W., Myers, G. J., Cox, C., Axtell, C., Shamlaye, C., Sloane-Reeves, J., Cernichiari, E., Needham, L., Choi, A., Wang, Y., Berlin, M., & Clarkson, T.W. (1998). Effects of prenatal and postnatal methylmercury exposure from fish consumption on neurodevelopment. *The Journal of the American Medical Association*, 280 (8), 701- 707. Retrieved from <http://jama.jamanetwork.com/article.aspx?articleid=187900>

Duzgunes, N. (1993). Membrane Fusion Techniques. Retrieved from: <http://books.google.com/books?>

id=K9LyXYSSIREC&pg=PA220&lpg=PA220&dq=diameter+of+an+endosome+1000+nm&source=bl&ots=usHL8udP62&sig=YAN9949nCn\_GgLFg6GKMJYmU4YE&hl=en&sa=X&ei=zAVXU5KYFZJJsQSw3oLQCw&ved=0CEkQ6AEwBA#v=onepage&q=diameter%20of%20an%20endosome%201000%20nm&f=false

Freeman, M. R. (2006). Sculpting the nervous system: glial control of neuronal development. *Current Opinion in Neurobiology*, 16, 119-125. Doi: 10.1016/j.conb.2005.12.004.

Greisbauer, L. (2007). Mercury contamination in fish and shellfish. *ProQuest*. Retrieved from <http://www.csa.com/discoveryguides/mercury/review4.php>

Kaur, P., Aschner, M., & Syversen, T. (2011). Biochemical factors modulating cellular neurotoxicity of methylmercury. *Journal of Toxicology*, 2011. <http://dx.doi.org/10.1155/2011/721987>.

Lebel, J., Mergler, D., Branches, F., Lucotte, M., Amorim, M., Larribe, F., & Dolbec, J. (1998). Neurotoxic effects of low-level methylmercury contamination in the amazonian basin. *Environmental Research*, 79, 20-32. Retrieved from <http://www.sciencedirect.com/science/article/pii/S0013935198938464>.

Leong, C.C., Syed, N.I., & Lorschieder, F.L. (2001). Retrograde degeneration of neurite membrane structural integrity of nerve growth cones following in vitro exposure to mercury. *Neuroreport*, 12 (4), 733-737. Retrieved from <http://www.ncbi.nlm.nih.gov/pubmed/11277574>

Mergler, D., Anderson, H.A., Chan, L. H. M., Mahaffey, K. R., Murray, M., Sakamoto, M., & Stern, A.H. (2007). Methylmercury exposure and health effects in humans: A worldwide concern. *AMBIO: A Journal of the Human Environment*, 36 (1), 3-11. [http://dx.doi.org/10.15179/0044-7447\(2007\)36\[3:MEAHEI\]2.0.CO;2](http://dx.doi.org/10.15179/0044-7447(2007)36[3:MEAHEI]2.0.CO;2).

Morris, R.L. (2014). Neurobiology Bio324 Primary Culture Of Chick Embryonic Peripheral Neurons 1: DISSECTION. Retrieved from [http://icuc.wheatoncollege.edu/bio324/2014/morris\\_robert/BIO324\\_Lab\\_Proc\\_1\\_Dissection\\_2014.htm](http://icuc.wheatoncollege.edu/bio324/2014/morris_robert/BIO324_Lab_Proc_1_Dissection_2014.htm)

Morris, R.L. (2014). Neurobiology Bio324 Primary Culture Of Chick Embryonic Peripheral Neurons 2: OBSERVATION of LIVE UNLABELED CELLS. Retrieved from [http://icuc.wheatoncollege.edu/bio324/2014/morris\\_robert/BIO324\\_Lab\\_Proc\\_2\\_ObserveLiveCells\\_2014.htm](http://icuc.wheatoncollege.edu/bio324/2014/morris_robert/BIO324_Lab_Proc_2_ObserveLiveCells_2014.htm)

Morris, R.L. (2014). Neurobiology Bio324 Primary Culture Of Chick Embryonic Peripheral Neurons 3: STAINING and OBSERVATION of LIVE CELLS. Retrieved from [http://icuc.wheatoncollege.edu/bio324/2014/morris\\_robert/BIO324\\_Lab\\_Proc\\_3\\_StainAndObserv\\_2014.htm](http://icuc.wheatoncollege.edu/bio324/2014/morris_robert/BIO324_Lab_Proc_3_StainAndObserv_2014.htm)

Mukherjee, S., Ghosh, R.N., & Maxfield, F.R. (1997). Endocytosis. *Physiological Reviews*, 77, 759-803. Retrieved from <http://physrev.physiology.org/content/77/3/759/>.

Reig, N. & van der Goot, G. (2006). Toxins in the endosomes. In I. Dikic (Ed.), *Endosomes* (pp. 145-152). New York, NY: Springer. [http://dx.doi.org/10.1007/978-0-387-39951-5\\_12](http://dx.doi.org/10.1007/978-0-387-39951-5_12)

Steuerwald, U., Weibe, P., Jorgensen, P. J., Bjerve, K., Brock, J., Heinzow, B., Budtz-Jorgenson, E., & Grandjean, P. (2000). Maternal seafood diet, methylmercury exposure, and neonatal neurologic function. *The Journal of Pediatrics*, 136 (5), 599-605. doi: 10.1067/mpd.2000.102774.

Purves, D., Augustine, G. J., & Fitzpatrick, D. (2001). Neuroglial cells. *Neuroscience-NCBI Bookshelf*. Retrieved from <http://www.ncbi.nlm.nih.gov/books/NBK10869/>

**I have abided by the Wheaton College Honor Code in this work.**

*Dana MacDonald*