ARTICLE
Using Chick Forebrain Neurons to Model Neurodegeneration and Protection in an Undergraduate Neuroscience Laboratory Course

Joseph R. Burdo
Biology Department, Boston College, Chestnut Hill, MA, 02467

Since 2009 at Boston College, we have been offering a Research in Neuroscience course using cultured neurons in an *in vitro* model of stroke. The students work in groups to learn how to perform sterile animal cell culture and run several basic bioassays to assess cell viability. They are then tasked with analyzing the scientific literature in an attempt to identify and predict the intracellular pathways involved in neuronal death, and identify dietary antioxidant compounds that may provide protection based on their known effects in other cells. After each group constructs a hypothesis pertaining to the potential neuroprotection, we purchase one compound per group and the students test their hypotheses using a commonly performed viability assay. The groups generate quantitative data and perform basic statistics on that data to analyze it for statistical significance. Finally, the groups compile their data and other elements of their research experience into a poster for our departmental research celebration at the end of the spring semester.

Key words: viability assay, cell culture, developmental biology, stroke, oxidative stress, dissection, journal club, poster presentation, concept mapping

Student-centered, investigative laboratories are known to generate more research interest in the students, and produce substantial learning gains (Myers and Burgess, 2003; Hurd, 2008). In our Research in Neuroscience course at Boston College, the students generate hypotheses based on the literature to test potentially neuroprotective compounds that have never before been investigated in our model of neurodegeneration. Previously, we have used a transformed mouse neuronal cell line (HT22, courtesy of Dave Schubert, The Salk Institute for Biological Studies) to develop a cell culture model of stroke. In an effort to include more developmental biology in the course and to make the laboratory more engaging and physiologically relevant, we have recently begun using primary chick forebrain neurons (CFN) isolated by the students from embryonic day 8 (E8) chicks in our model. They are easy to isolate, are biochemically and morphologically similar to mammalian primary neurons, and their use avoids many of the ethical concerns that go along with isolation of primary neurons from rats or mice, the typical mammalian models used in primary neuronal culture.

The goals of the course are to:

1. Build group work and organizational skills
2. Continue and extend the neuroscience education the students have received in previous courses
3. Teach sterile cell culture to the students, a technique used in many academic and industrial neuroscience research laboratories.
4. Teach basic principles of statistics in an applied setting
5. Build student oral presentation skills through journal club and poster presentations.
6. Involve the students in a realistic and authentic research experience

COURSE OUTLINE

The Research in Neuroscience course is a standalone laboratory course serving 12 students per spring semester at Boston College. Typically, the majority of the students in the course are Biology majors. The prerequisite for this course is the Introduction to Neuroscience course that I teach in the fall semesters, which is predominantly made up of junior and seniors, so many students in the lab course have recently had an introduction to the general principles of cellular and molecular neuroscience. In addition, Cell Biology is a prerequisite of Introduction to Neuroscience. All Biology majors at Boston College are required to take Investigations in Molecular Cell Biology, a laboratory based course that introduces them to basic techniques such as pipetting and proper microscope use (which are critical in the Research in Neuroscience course), as well as more advanced techniques such as immunoblotting, restriction digests of plasmids and PCR.

In this course, students work in groups of three that are determined by the instructor. One of the benefits of Introduction to Neuroscience being a prerequisite to this Research course is that I know the students relatively well, including their strengths and weaknesses, by this point. Group formation does not take place until the second or third meeting of the course, which gives me time to refresh my memory from previous semesters as to the capabilities of each student, and place them into groups wherein the students have complementary skills. For example, there is a substantial amount of quantitative work involved in the class when calculating cell plating concentrations, molar ratios, chemical compound and media dilutions, and analyzing cell viability assay data. I will place at least one student into each group who is comfortable working with quantitative calculations and use the power of peer instruction during the class sessions to allow the students to learn from each other, which has been shown to

produce more substantial learning gains than solely instructor directed learning (Crouch and Mazur, 2001; Nicol and Boyle, 2003). During the semester, the students will learn several new skills such as mammalian sterile cell culture and lactate dehydrogenase (LDH) viability assays, and these techniques are supported by some that they have learned previously, including pipetting and microscopy as described above. The results from their experiments are shared with other instructors and their peers during our annual Biology Research Celebration at the end of the spring semester.

COURSE MECHANICS

The three credit hour course runs for a full 15-week semester, and is scheduled for two, three hour blocks per week. Before the semester begins however, the students are informed via email that, similar to a "real" research setting, it's not possible to perform experiments twice a week for three hours and expect to get meaningful data, nor is it possible to get a sense of how true research works in a laboratory setting. The time commitment expected from them is still roughly the same as if they did attend class during the scheduled period, but this commitment is spread out throughout the week. They are also reminded of this during the first class session so they can drop the course before the drop/add period ends if they have objections to the policy. To date, we have not had any students drop the course for this reason.

During the first meeting of the semester, we first discuss the syllabus as a group. This includes the course outcomes and unique scheduling requirements as described above. The semester schedule as listed in the syllabus is purposefully vague in many regards. I tell the students the progression of the experiments depends largely on their preparation, the skills that they acquire throughout the semester, hard work, and to a not insignificant extent, luck. I again emphasize how it is difficult to plan out experiments in an academic research laboratory more than a week or two at a time, since the next step experiments depend upon the success of the previous experiment, and also if the results are consistent or not with the hypothesis going into the experiment. The students are told that as the semester progresses, we will set goals for each group on a weekly basis.

Even though the weekly schedule is not determined ahead of time, there are end of semester goals they are working towards that are discussed in the syllabus. In this course, the students will be using their primary neuronal culture skills to grow and maintain CFN in culture, treat them with an empirically determined concentration of H_2O_2 to mimic the oxidative stress present in stroke and many other neurodegenerative diseases, and find a dietary antioxidant compound that they hypothesize will protect against that toxicity. This determination of toxicity and its prevention by dietary antioxidants is the main activity in the course, and the one which all of our other dependent tasks (such as learning primary cell culture) revolve around. The students are provided with a review article discussing the effects of H_2O_2 and other oxidative stressors in neurons (Lo et al., 2003), and are encouraged to seek out other publications to augment the review. The cellular and molecular mechanisms of toxicity are discussed throughout the semester, so the students do obtain a good understanding of its basis.

The students are given a copy of a Methods in Cell Biology chapter coauthored by Steve Heidemann of Michigan State University, who has worked out much of the CFN isolation and culture mechanics to make it feasible to perform this work in an academic laboratory course setting (Heidemann et al., 2003). This chapter condenses much of the previous 50 years of CFN literature and presents a broad guide for those isolating and growing CFNs for the first time. Since the chapter does go into a substantial amount of background and presents alternative options at several steps of cell isolation and culturing, I have used it as a reference to prepare a step by step outline for my students to use when they are isolating and culturing their CFN (Supplementary Document 1). The students also receive a document of cell culture basics from Gibco, and we watch two short videos in the first class that introduce cell culture and discuss the requirements for sterile mammalian cell culture (http://www.invitrogen.com/site/us/en/home/References/gibco-cell-culture-basics.html).

The students are required to keep a detailed laboratory notebook including their thoughts, readings, hypotheses, experiments, calculations, results, and interpretations. We use a lab notebook guide based (with permission) on an excellent document developed by Brett Couch in the Biology program at the University of Minnesota (http://blog.lib.umn.edu/jveldof/calculator/BiologyLaboratoryNotebooks.pdf). The guide contains a rubric so the students know exactly what they will need to put into their notebook to achieve a specific grade (Supplementary Document 2). We use notebooks with carbon copy pages so the students can leave the notebook itself in the laboratory and still take specific pages home with them if needed. This has worked well for us for several reasons. One, the students work in groups of three, and divide weekly responsibilities between themselves. Because of the need for flexibility with the experiments and the students' busy schedule, I don't require them all to be in the lab for every step, as long as each member at some point in a few weeks' work does participate in all of the techniques that we use. In practice, we have not had a problem with a student not doing his or her own fair share of work. In general, they appreciate the need for flexibility in the lab and take on the responsibilities professionally and with enjoyment. Since they are not necessarily all physically participating in each step along the way, it's important that they talk to the other members of their group outside of lab and have a documented record of what did take place when they were not present. The carbon copies of their lab notebook pages allow them to share the details with others in their group outside of lab if necessary. We also require that the lab notebooks remain in the laboratory so they don't get lost or forgotten, as well as for informal evaluation. Every Friday, the lab TA or I collect the notebooks and spend a few minutes on each reviewing the notes from that week. One of us is almost always in the lab with the students while they are working on their

experiments, so we have a good general idea of what they have been working on, and any roadblocks or pitfalls they may have encountered along the way. Particularly early on in the semester, we will help them with the many calculations they are required to perform in the course of their weekly lab duties, and are constantly reviewing their written steps with them in their notebooks. However, later in the semester when the students are working more autonomously, the weekly notebook checks are important to let them know that they are still required to keep detailed and accurate notes or their work, even as much of it becomes routine to them.

Once a week throughout the semester, one of the students will present a paper in a journal club style format to the rest of the class. The requirements for the paper are that it is a primary research publication, and that it has something to do with stroke research, either *in vivo* or *in vitro*. In preparation for each presentation, each student is responsible for reading the article and constructing one concept map of the introduction and one of the figures in the paper. Concept mapping is a flowchart like procedure used to illustrate a progression of thoughts and ideas (see Novak and Gowin, 1984, for review). It helps students visualize complex processes, and their use can improve problem solving skills and meaningful learning (Rendas et al., 2006). A number of concept terms, which are typically individual words or short phrases, are connected together using linking arrows and phrases to indicate directionality of idea flow. I have the students use 15-20 concepts taken from the introduction to create a map which encompasses the background to the current research from the paper, as well as a lead in to what the authors will be doing in their work. I also have them construct a separate map using terms they generate from just the figures. This map should walk the viewer through the actual work that was done by the authors to test their hypothesis. I have found that these concept maps are a good way to ensure the students are reading and understanding the information in the paper. They don't take very long to review for each student (a 20-term map can be understood within one or two minutes if it is well constructed) and I typically grade them solely for completion as long as it is evident that the student did read the paper and put a reasonable amount of thought into their construction. During our first class meeting, the students are shown how to construct a basic concept map using the whiteboard in our room. They are also shown how difficult it can be to construct an easily understandable map using pen or pencil and paper, since the concepts and their linkages tend to change as the map is being constructed and the student is thinking through the material. I have my students use software called CMapTools, from the Florida Institute for Human and Machine Cognition (IHMC, http://cmap.ihmc.us/). This software is free and available on several different platforms. It allows the students to type in concepts and linking phrases, and connect them in a drag and drop manner. The maps can be saved in a native format and uploaded to an IHMC housed server for anyone (or a defined set of users) to view, or they can be saved locally and emailed in several different formats. I typically have my students send them to me in a PDF format, as that is the easiest to view and markup electronically if I want to provide feedback to the students.

I also require the students to create a Google account if they don't currently have one. We use Google Calendar to coordinate journal club presentation dates and to sign up for equipment in the laboratory, such as the cell culture hoods and the spectrophotometer. This has worked better for us than paper calendars. Most if not all of the students consistently carry some type of connected type of device with them which allows for web page display and interaction, so they don't need to be located within the building to make quick additions or changes to their calendar.

All of the students in the course have previously learned to use an air displacement micropipettor. However, many of them have not done so for up to two years, so it is important to provide them with an opportunity to refresh their skills before they proceed to the culturing of our neurons. The students also need to be comfortable with a powered serological pipetor, which we routinely use in the culture hood. I provide the students with a copy of a training guide during our second lab meeting and have them work individually, rather than in groups, so all of the students perform all of the exercises. We predominantly use 5 and 10 ml serological pipets with the powered pipetor, as well as 100-1000 ul, 10-100 ul and 2-10 ul micropipettors. For the training exercises, the students are required to aspirate water from a beaker and pipet it into weigh boats, weigh their sample, and then record it. They do this for two different volumes, typically at the top of the adjustment range and in the middle, for each of the micropipettes, and they repeat each volume several times. I use this exercise to discuss the difference between accuracy and precision, and to show to them that the accuracy can vary depending upon where they are in the volumetric range of the pipet. I also have the students use the repeat pipetting technique to weigh water from each pipetor set in the middle of its range in a similar manner as described above. Repeat pipetting involves depressing the plunger to the second stop and holding, inserting the tip into the fluid, and then releasing the plunger. More than the indicated value is aspirated into the tip (this is why repeat pipetting can't be used at the top end of the adjustable range), but if the plunger is depressed only to the first stop instead of the second when dispensing, the indicated volume is accurately delivered. If the plunger is held down to the first stop after this delivery and placed back into the fluid to be pipetted out, many repetitions of fluid can be dispensed relatively quickly without introducing air bubbles into the receptacle. This reduces variability when pipetting small amounts of biological solutions containing proteins that tend to cause bubbling or foaming (Suominen, 2009).

Before the third course meeting, the students are provided with a copy of our embryo dissection guide. During the next several meetings, the students will be practicing their dissection skills, so it is important that they read and understand the procedure ahead of time. At the beginning of the third meeting, I will show the students the

standard aseptic hood setup in which they will be dissecting the embryos as well as culturing and maintaining the isolated neurons. We use laminar flow hoods that do not have glass drop down fronts to them (Figure 1), which makes them easier to use with dissection microscopes, but they are still suitable for sterile cell culture if the students take care not to lean into the hood and disrupt the proper air flow when working. If these types of laminar flow hoods are not available at your institution, it is possible to perform the dissections using portable bench top flow laminar flow hoods, which take up much less space and are a fraction of the cost of full sized units. Our hoods are large enough to accommodate two students working side by side, and my students tend to enjoy and find beneficial working in pairs when performing the dissections or working with the cultured neurons, particularly early in the semester when they are learning the proper work flow and improving their technique. However, this tends to increase the likelihood of talking into the hoods, another source of potential contamination. Theoretically, this is one of the biggest challenges when working with cell culture novices, but in practice, we have not had significant problems with student derived contamination in our CFN cultures. Our use of transformed HT22 cells in the past with the stroke cell culture paradigm did typically result in at least one significant student derived contamination event per semester. This most likely arose because of the continual passaging of the same initial culture of cells, which is now avoided by our use of newly isolated CFNs for each experiment.

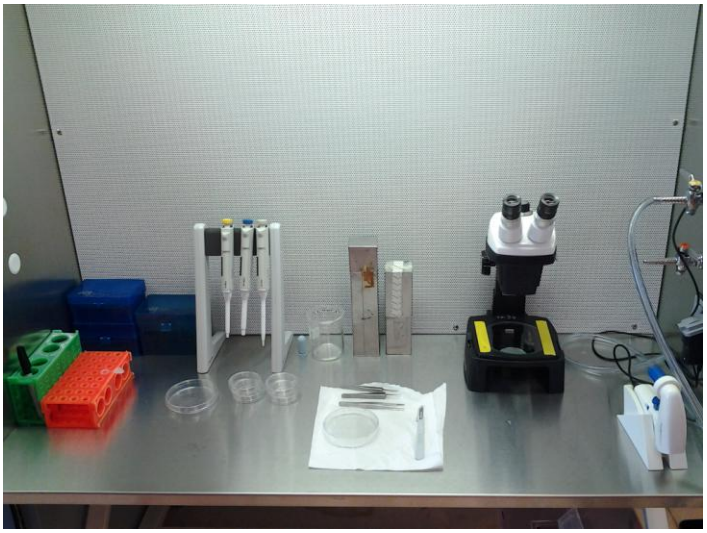

Figure 1. Laminar Flow Hood Setup for Chick Forebrain Neuron Dissection

After showing the aseptic set up to the students, I demonstrate the egg preparation and embryo dissection to the entire group, as described in depth in Supplementary Document 1. While eggs sourced locally may be ideal to use for derivation of chick forebrain neuron cultures, there are no farms in the vicinity of Boston College that sell fertilized eggs in suitable numbers for our purposes. We have used several sources of eggs in the laboratory, with the best to date coming from www.mypetchicken.com. This reseller is an intermediary for several moderately sized poultry farms around the country, and they make available hatching eggs for education with free Priority Mail shipping via USPS. This keeps transit time to a minimum. Unfortunately, UPS and FedEx will not ship fertilized eggs. I have found that it is critical to have the package of eggs held for pickup at the post office itself, particularly if you have them shipped to your home. The eggs will retain their greatest potential fertility if they are removed from the hen's nest within 24 hours of laying (to reduce extended time at elevated temperatures) and kept between approximately 10 and 24°C during shipping. Most farms that sell eggs for hatching will follow these guidelines to the best of their ability, including shipping the eggs with cool packs during the hot summer months. If they are delivered to your door and sit in the cold or sun, fertility may be greatly reduced. If the eggs are sold as fertilized but suitable for consumption and not hatching, they are typically refrigerated at temperatures below 10°C after being removed from the hen, which negatively affects fertility.

After the eggs arrive, it is important to let them rest for a minimum of 24 hours with the large end up at a controlled temperature. We use a small refrigerator with a temperature controller (A19AAT-2C, Johnson Controls, Milwaukee, WI) to maintain the eggs at approximately 15°C after arrival. In practice, we have found that the eggs can rest at this temperature for 7-10 days after arrival with only a minimal impact on fertility. This is beneficial, as it allows us to order several dozen eggs at a time and stagger their timed fertility to coincide with the dates they will be used in the lab.

CFN are typically harvested at embryonic day 8 (E8). At this stage of development, there are no glial cells in the brain, reducing the potential for non-neuronal contamination, and there are no functional pain sensing pathways. This makes the isolation and culturing relatively easy compared to primary neurons from mammalian sources such as rat or mouse, and reduces the ethical concerns associated with their use. Avian embryos are not considered to be live animals by US regulatory agencies, and most IACUC committees only require an animal use protocol to be assembled and followed if the embryos are used after E13.

To allow the eggs to develop after storage in our 15°C refrigerator, we place them in a repurposed cell culture incubator dedicated for egg incubation. Inside of this incubator is an egg rocker (Automatic Egg Turner, www.mypetchicken.com), which slowly rocks the eggs back and forth (again, large end up) to mimic the movement that would be provided by the hen. The incubator is maintained at 37.5°C and humidified to approximately 80% using a beaker of water in the lower part of the chamber. Higher levels of humidity can be detrimental to early embryonic development, so using a container of water with a smaller surface area than would be typically used for culturing cells at near 100% humidity is beneficial for the eggs. If a dedicated incubator is not available, there are smaller, relatively inexpensive Styrofoam incubators with electric heat elements and water trays for humidity that accept the egg rockers (Hovabator

Genesis Incubator, www.mypetchicken.com). Another possibility is using the same incubator within which the isolated neurons are maintained. The cell culture media we use in the course is non-CO_2 dependent (described below), allowing for co-incubation of eggs and cells in the same chamber if one is careful to avoid contamination. In our hands, we typically obtain 50-75% success rate for E8 embryos, which is quite good considering that the farms will state there is no guarantee for any particular value of hatching success due to the uncertainties of ambient temperature, shipping conditions and transit time, and incubation conditions before use. In our experience, the 24-hour period of cool incubation and the controlled warm incubation to E8 provided by the use of the carefully controlled cell culture incubator is critical to obtaining maximum fertility.

There are two points that I feel are the most important to emphasize for a successful dissection. One, all traces of meninges must be removed to keep fibroblast and endothelial cell contamination to a minimum. The meninges sometimes come off easily during initial removal of the telencephali, but other times great care must be taken using the dissecting microscope to ensure this. If done properly, the meninges will come off as a sheet of tissue from the exterior of the telencephali. Once they are removed, the pink to red color of the brain as seen under the dissecting microscope will become off white. A common phrase heard relating to these dissections is "never a trace of red" for the tissue being plated out. However, this can be somewhat misleading, as there is microvasculature inside of the telencephali themselves at E8, which appears as small dots of red under greater magnification with the dissecting microscope. These vessels are not practical to remove, which invariably leads to some minimal level (<=5%) of non-neuronal cells in the cultures. Another important point to emphasize is the need for minimal contamination of the embryo with the egg yolk. The yolk contains many microorganisms which will overwhelm the neuronal cultures if care is not taken. The students use inverted light microscopes at 400x total magnification to visually scan for contamination in their cultures, and they are shown by the instructor what typical microbiological contamination looks like towards the beginning of the course for comparison purposes. The yolk seems to be a far greater and more likely source of potential contamination than microorganisms introduced from poor student technique while plating or maintaining the cells. There are many bacteria present in the yolk, but there also a large amount of eukaryotic contaminants including protozoa in the eggs that we typically work with. This can vary from farm to farm and even chicken to chicken, but in the three separate farms we have used for eggs, we have never seen yolk that is completely free of contaminants. Fortunately, the microorganisms that we have encountered that are endemic to the egg do not appear to be detrimental to neuronal growth, and they can be minimized by several steps. One is good technique when cracking the egg and removing the embryo to ensure minimal yolk contamination. This takes practice, but after a few eggs, most students are able to accomplish this task. We also rinse the embryo and brain at several stages of the dissection using filters that fit into 50ml tubes (Fisher Scientific, product # 22-363-548). Multiple 60mm dishes with fresh buffer are used as the dissection progresses to dilute and wash off any contaminants. Finally, the dissection instruments are continuously soaked and rinsed in 70% ethanol as the dissection progresses.

We culture our neurons in Leibovitz L-15 medium (Invitrogen, San Diego, CA), predominantly due to its ease of use and robustness of results during our growth period of interest. L-15 media is non-CO2 dependent, being buffered by its amino acids instead of sodium bicarbonate. CO_2 independence is beneficial, as it makes it easier to find a suitable cell culture incubator, needing only stable temperature and humidity control. Also, early on in the development of their skills, the students tend to keep the dissected tissue and isolated cells in the cell culture hood for long periods of time. Cells incubated in CO_2 buffered media may suffer because of this, but the L15 media keeps pH changes to a minimum. For the first two days after plating, the L15 is supplemented with 10% FBS (Atlanta Biologicals, Atlanta, GA). After two days, the old media is aspirated off and replaced with L15 containing N9 supplement (Heidemann et al., 2003). We switch to serum free media primarily because serum interferes with the lactate dehydrogenase (LDH) viability assay that we use to determine cell health after using our cell culture model of stroke and neuroprotection (described below). Alternatively, if the students are sufficiently skilled in the chick brain dissection and the cells are not kept in the room air for an excessive period of time, Neurobasal medium with B27 (Invitrogen, San Diego, CA) or N21 (R&D Systems, Minneapolis, MN) supplement can be used in a CO_2 incubator. The benefit of this culture system is that the neurons can be plated in serum free media. This may be important for those that want or need to use a completely defined culture medium, and it also avoids the need for a complete change of the media after two days of growth in FBS containing media. Removing all of the media from a plate well by students not yet skilled in the process can lead to dehydration and reduced viability or death of the neurons. It also promotes aspiration of cells from the portion of the well bottom closest to the vacuum source if care is not taken. If the same media is used throughout the life of the culture, ½ to ¾ of the well volume can be aspirated gently and replaced with new media. This minimizes or eliminates cell aspiration, and when we have tried this, the remaining waste products and cell metabolites do not seem to negatively affect cell viability. We have attempted to use L15 media with N21 or N9 supplement and no FBS during the first two days after plating, but cell viability as judged by morphological changes is diminished beyond what is acceptable to us.

After the cells have been in culture for five days, they typically possess substantial numbers of neurites (Heidemann et al., 2003). In our course, the students are using H_2O_2 as an oxidative stressor to mimic a stroke-like insult. Phasic changes in oxygen concentration during and after a stroke are known to lead to increases in oxidative stressors, including H_2O_2, and this stress can directly lead

to cell death (reviewed in Lo et al., 2003). At 5 DIV, the students replaced the growth media with that containing either 500, 750 or 1000 μM of H_2O_2. I give them these concentrations as a starting point for the toxicity testing so they can learn how to generate a dose response toxicity curve and learn about some pharmacological properties of chemicals, including LD_{50} (and later, ED_{50}).

To measure changes in viability during our experiments, we use the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). This assay measures release of LDH, which is a cytosolic enzyme that participates in the conversion of pyruvate to lactate. LDH is a ubiquitous enzyme and its release into the extracellular space has been used for decades as a marker of viability in numerous cells, including primary cortical neurons (Koh and Choi, 1987). We followed the full protocol as provided by the manufacturer, but briefly, after a 24-hour period of H_2O_2 treatment, an aliquot of cell culture media is removed from the cell culture wells. The cells are then frozen and thawed to kill all the cells, and the media is sampled again. The pre-freeze values indicate the amount of LDH released due to the specific culture conditions, while the post-freeze values indicate the total amount of LDH present in the cells. While the students do theoretically plate out the neurons at the same concentration per well, in practice this value can vary by as much as 10% due to numerous factors such as settling of the cells in the master tube and pipetting errors. Also, it may be the case that the specific treatment conditions affect the amount of LDH present in the cells and not just its release. Expressing the amount of LDH released by the culture conditions as a percentage of the total amount of LDH normalizes these potential changes.

For the viability assays, a 48 well plate format works best for us. We have found that it provides a good balance between a large *n* per plate for our control cells and each experimental condition we are testing and a large enough well diameter for the students to work with easily. We typically plate the chick forebrain neurons at 2.5 – 3.0 x 10^5 cells / cm^2. If the neurons are plated too far below this value, neurites won't develop to their fullest extent, leading to deficient neuronal morphology. While a higher cell density may not be expected to negatively impact the colorimetric LDH assay, and may even improve the signal to noise ratio, there are several reasons to avoid cell numbers much above this value. One of our expected student outcomes from the course is an increased knowledge of neuronal morphology. The students use inverted light microscopes at 100x-400x total magnification to view their cells, and (using an attached digital camera and computer) to produce micrographs for their notebooks and the end of year poster presentations. If the neurons are plated too densely, it becomes difficult to discern individual cells and their processes. It also makes it more difficult to detect the contaminating prokaryotic and eukaryotic organisms. Finally, at least with one particular neuronal cell line it is known that cell density and the toxic response to oxidative stressors are inversely related (Maher, 2001).

After incubation with the LDH assay reagents in a 96 well plate, the samples are read at 490 nm in a spectrophotometer (SpectraMax M5 plate reader, Molecular Devices, Sunnyvale, CA) and the data is exported to Microsoft Excel. Media blanks are subtracted to account for the absorbance of light in the media at 490 nm, the relative LDH release is calculated as described above, and the data is plotted as a percent of control in bar graph format.

After performing LDH assays a minimum of three times to assess viability (Figure 2), each group compiles their data into one Excel spreadsheet to study the overall trend of the H_2O_2 effect. They learn how to plot the mean effect on a bar graph for the experimental groups and appropriate controls, generate error bars from standard deviation and standard error of the mean values that they calculate (and learn the difference between the two in the process), and perform basic tests of statistical significance including ANOVA and post-hoc T-tests. After compiling and analyzing this data, they determine what concentration of H_2O_2 works best in their hands to test potential neuroprotection by dietary antioxidants.

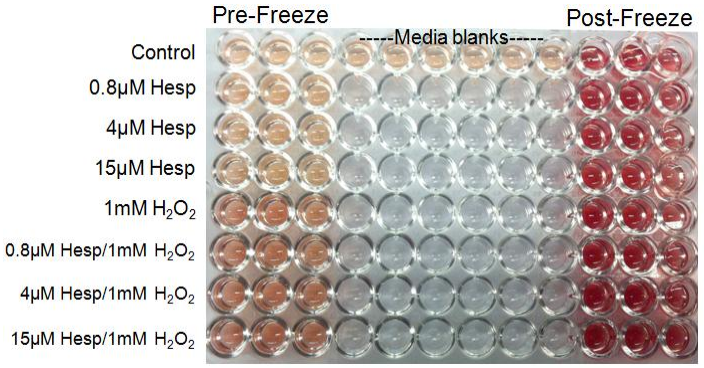

Figure 2. Typical LDH Assay Microwell Plate. A darker color in the media indicates a higher concentration of LDH, thus a greater amount of neuronal death. Freezing of the cells allows for maximal cell death determination.

Many of the papers that the students present during our weekly journal club meetings pertain to *in vitro* and *in vivo* studies of compounds in grains, fruits and vegetables that have antioxidant capabilities. As we discuss during the first few meetings of the course, there are very few options for treating people that have suffered a stroke. Tissue plasminogen activator (tPA) is the only compound that's approved to treat those who have undergone a thrombotic or embolytic stroke, but it is only beneficial if the patient makes it to a hospital within three hours of the stroke onset. This is typically not the case, particularly for mild to moderate stroke when the patient may not even know that he or she is having an attack. Also, tPA can lead to hemorrhaging in those with an initial ischemic attack (NINDS, 1995). Furthermore, tPA is not a directly neuroprotective compound; it primarily serves to remove the clot that has obstructed blood flow. Thus, the search for other compounds that are protective before or after a stroke is one of utmost importance. Many students in the current generation are very health conscious, and are naturally curious about the molecular composition of their

food. Therefore, we focus solely on natural antioxidants found primarily in fruits and vegetables to test as potentially neuroprotective compounds in our *in vitro* model of stroke. As the groups are becoming more comfortable with the neuronal culture and generating dose response data with the hydrogen peroxide toxicity, they are required to identify three different dietary antioxidants that they would like to test. To do this, they must first determine that the compounds they are interested in have never been tested in our system before. Essentially, they must be generating new data and not just repeating studies that have been performed previously. The search strategy typically involves scanning PubMed and Google Scholar using keywords that relate to the antioxidant in question and our experimental paradigm. For instance, a group may choose to search for "fisetin AND chick neuron AND hydrogen peroxide" to begin to identify previous work that has studied the effects of fisetin in our culture system. That particular search may identify work that has been done *in vivo* with chick brains or *in vitro* with neurons other than telencephalic cells, but that can typically be determined quickly by reading the abstracts. The students also need to predict if the effects of fisetin and H_2O_2 interact at some level within neurons. Maybe previous work has shown that fisetin increased the activity of catalase in primary rat hippocampal neurons. From their previous reading and discussions, the students would know that catalase is one of the enzymes responsible for converting hydrogen peroxide to water, thus detoxifying it. I would require the students to attempt to determine if catalase is present in chick telencephalic neurons, but since these cells are not as commonly used as mammalian cells, the information may not exist. This can be used as a launching point for discussing how much is still unknown in the realm of biology and neuroscience, the benefits and pitfalls of using non-human animals and cells as models of human disease, and also the evolution of nervous systems.

Once the groups have identified three compounds of interest, they forward them on to me so I can double check the uniqueness, and determine if any of them can be ordered synthetically. Typically, Sigma-Aldrich (St. Louis, MO) is our source for compounds. The price for those compounds that are of interest to the students typically ranges from tens of dollars to several hundred dollars for a mass sufficient to use in several experiments. I have the groups identify three compounds of interest since some of the most current research has not trickled down to the synthetic market yet, and we try to avoid purchasing compounds that are more than $100.

After purchasing one of the compounds per group, the students use the existing data in the literature to develop a hypothesis of what concentrations to test against H_2O_2 toxicity in our neurons. This can be challenging for them if the previous data was not generated in neural cells or if H_2O_2 was not the stressor used. For instance, the students may be interested in testing the effects of the flavonoid hesperetin in our system, but it may be the case that previous *in vitro* work has only investigated its protective effects against LPS toxicity in immune cells. The students will have to take into account the culture system of the previous work, how the hesperetin was delivered to the cells, and any common downstream cellular effects between LPS and H_2O_2 to determine three concentrations of hesperetin to test. After this has been determined, the groups will generate a dose response curve for the protective compound in much the same way they did for H_2O_2 alone. If initial experiments show no protection, or similar protection at all three concentrations, I ask the students to reevaluate their concentration choices and pick another range (usually adjacent to their previous concentrations) to test.

By the end of the semester, each group typically has had time to run five or six experiments testing their neuroprotective compounds. If they happened to find a successful concentration range right away and their LDH assays work well to generate data with minimal variation, then I have them add to their *n* by running more experiments using the same conditions. This is not the usual case. Typically, the initial concentration range is not ideal and it needs to be tweaked, or even more commonly, the range may be sufficient but there are inconsistencies in the LDH data. This is actually a benefit in the class, because it leads us into discussions about when it is ok to exclude data from statistical analyses and when it is not ok. For example, I will usually tell the groups that if they have identified contamination or sparsity of cells in one individual well ahead of time and make a note of that in their notebooks, it may be rational (and ethically ok) to exclude that data point ahead of time, whether or not that well ends up being an outlier when their results are compiled. But if they don't notice anything wrong or different about an individual well of cells or the way that they treated it during the viability assay, and the data isn't consistent with that from their other wells or their hypothesis, it isn't ethical to exclude it just because it doesn't look right to them. This serves the benefit of making the groups take their time and cautiously examine all of their wells in the culture plates and proceed with their viability experiments slowly and methodically to keep problems to a minimum, and to prompt them to identify and make a record of problems when they do arise.

During the last week of classes at Boston College, the Biology department holds a Research Celebration Day where students can present posters of their research experiences to faculty and other students. Each group in the lab course is responsible for constructing a poster detailing their accomplishments, as well as their setbacks, to present during this poster session (Figure 3). The students typically enjoy this experience very much, as it gives them an opportunity to show off their hard work to their peers and other instructors in the Biology department that they have had as teachers in semesters past, and serves as a capstone experience for many of our graduating seniors. It also gives the students another glimpse of what a real research experience is like, as the posters they construct are very similar to what would be found at a regional or national neuroscience research conference. During this poster session, the focus is as much on what the students learned throughout the semester as on what their results show. Many of the

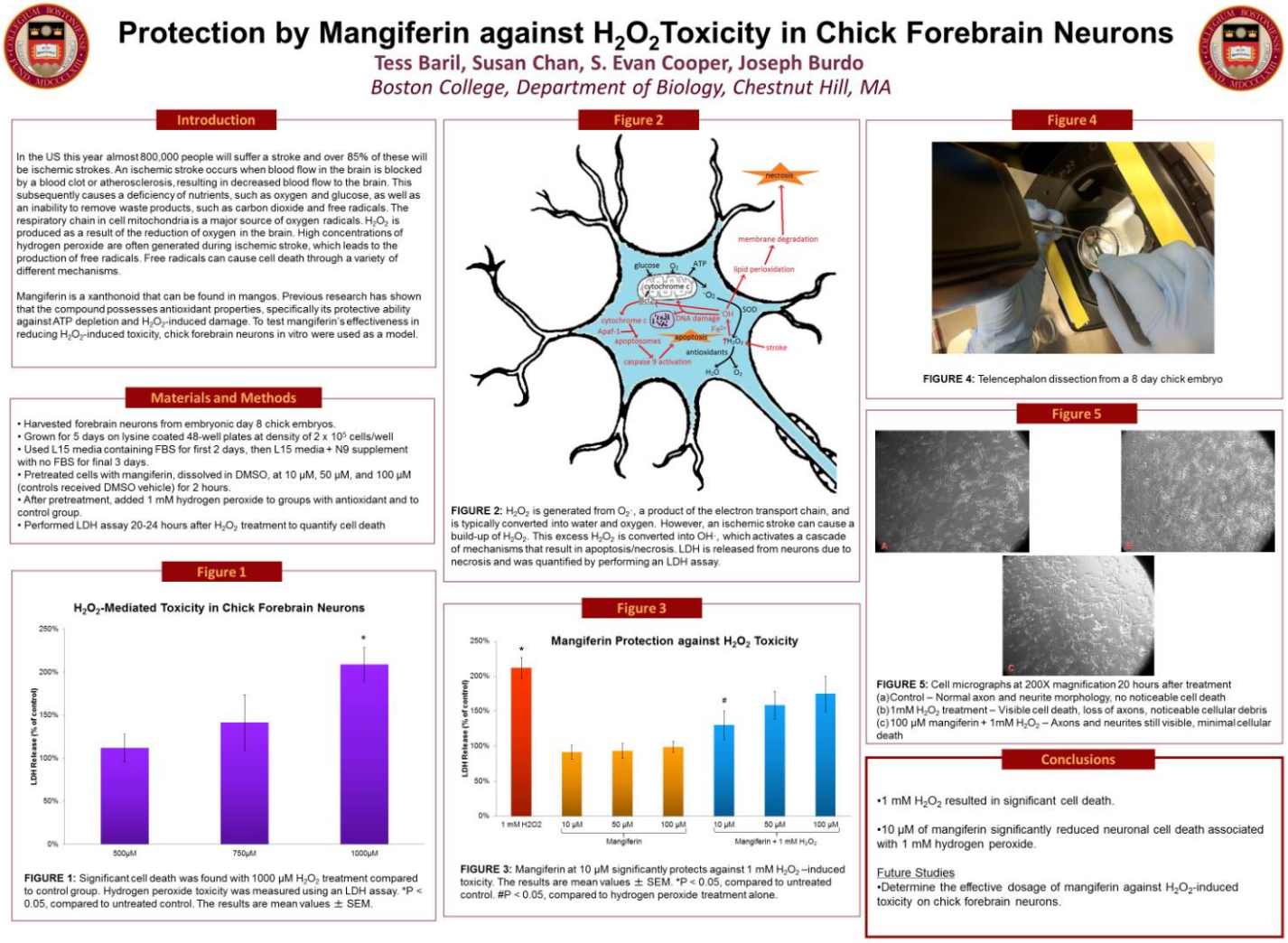

Figure 3. Typical research poster constructed for the Research Celebration Day from one group's data.

faculty want to know what stumbling blocks the students may have encountered along the way and what they did to work around them. I emphasize this to the students when they are analyzing their results and constructing their poster. Out of the four groups we have every semester, on average 50% of them may have shown that their compound is neuroprotective at the doses they tested. For the groups that have not found protection, they many times worry that their "negative" data is a disappointment and that their results are less valid than their peers who have found protection. Emphasizing to them that their data is not negative is critical; if their viability assays physically worked, and they showed no protection by their compounds, they are still advancing the field of dietary neuroscience by giving us new information on their compound in this particular toxicity paradigm. I believe that it is also critical to emphasize to the students how their problem solving skills have advanced over the course of the semester. I will keep notes on problems that each group has encountered and what they did to overcome those problems to keep their research moving forward. I use this information for grading purposes, but more importantly, I remind the students of these accomplishments at the end of the semester to reinforce their progression as young scientists during the four months we have had together in the lab.

SUMMARY

In closing, I believe that the laboratory course described herein provides an authentic and enjoyable research experience for our students, and can be easily adapted to suit different student experience levels and institutional capabilities. The initial design and planning of the course took many hours, but successive iterations of it can be implemented with the same time commitment as many other lab courses using the instructions provided throughout this article. At the beginning of the semester, approximately 5-10 hours are required to order and prepare supplies and determine the egg ordering and incubation schedule for the semester. During the semester itself, 2-4 hours per week in addition to the normal course period are required for tasks such as reviewing student notebooks and proposed papers for the journal club, preparing media and restocking supplies as needed. If

possible, the utilization of an undergraduate TA who has taken the course previously reduces the faculty time commitment and can be a great benefit to the students currently taking the course, as the TA will have more recent knowledge of how to avoid the pitfalls of learning the embryo dissection, primary cell culture and viability experiments for the first time. While the expense and the organizational work involved with using CFN instead of transformed cells is somewhat greater, the knowledge and enjoyment the students derive from the embryo dissections and use of primary neurons in authentic neurodegenerative research makes it well worth it. While not statistically rigorous, the student evaluations for this course seem to support this idea. On a 1 to 5 scale for the question "How would you rate this course overall?", the rating has increased from a mean of 4.49 for three semesters of HT22 cell use to a individual rating of 4.90 for the current semester when using the CFNs. Other than the embryo dissections and the cell type used, the research paradigm and mechanics of the course have remained constant. I would encourage those interested in involving their students in research that encompasses developmental biology, anatomy, physiology, neuroscience and nutrition all within one semester to use the experience described within and continue to improve upon it.

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Address correspondence to: Dr. Joseph Burdo, Biology Department, Boston College, 140 Commonwealth Ave, Chestnut Hill, MA, 02467. Email: joseph.burdo@bc.edu