ARTICLE
Using Cultured Mammalian Neurons to Study Cellular Processes and Neurodegeneration: A Suite of Undergraduate Lab Exercises

Rachel Catlin, Abigail Taylor, Lisa Ditchek, Samantha Burnett, Showkhin Khan, Olivia Todd, Marguerite Adams, Eva Touhey, Andrew Wynkoop, and James Ryan
Biology Department, Hobart and William Smith Colleges, Geneva, NY 14456.

Cell culture is a powerful tool for exploring cellular function. Culturing primary neurons has revealed how neurons communicate in learning and memory (Kandel, 2006) and provided insights into the mechanisms of neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Alberio et al., 2012; Trinchese, et al., 2004). Here we describe a series of four modular laboratory exercises to integrate this neuroscience technique in undergraduate teaching laboratories. First, we describe the modular approach. Then we provide educators with simple techniques for culturing rat primary neurons, performing immunohistochemistry to label cellular components, and illustrating neurodegeneration caused by reactive oxygen species. We describe teaching exercises that culminate in student-generated research projects. Finally, we describe potential barriers students may face when integrating modern cell culture experiments into teaching laboratories.

Key words: cell culture; neurites; axon path finding; growth cones; immunohistochemistry; free radicals; antioxidants; neurodegeneration

As detailed in AAAS's "Vision and Change" report (Brewer and Smith, 2011), undergraduate faculty are charged with implementing meaningful changes in the way we prepare the next generation of biologists/citizens who will have to navigate the implications of new biological discoveries. Consequently, many biology faculty have moved to inquiry-based teaching with an emphasis on student-centered learning. Engaging students in inquiry-based learning helps undergraduates learn important vocabulary, grasp science content, and master how to do science. (Allen and Tanner, 2003; Barkley et al., 2005; Brame et al., 2008; National Research Council, 2008).

In undergraduate laboratories, change has often been slower. Here instruction often still relies on "cookbook" experiments where instructors provide the questions, hypotheses, and experimental design. The real and meaningful change called for in Vision and Change must include how we teach students to do science. Toward that end, we describe a suite of modular, multi-week, research-focused neurobiology laboratories that use a simple neuron culture system.

These exercises expose neurobiology students to real-world hypotheses based on reviews of the primary literature. They also ask students to design their own experiments, interpret data, and report their findings in oral and written forms. The specific learning objectives are to:

(a) teach experimental techniques in neurobiology,
(b) build critical thinking skills,
(c) ask students to identify scientific hypotheses and design experiments to test them, and
(d) enhance scientific presentation skills.

At Hobart and William Smith Colleges, neurobiology laboratory consists of three four-week units: crayfish neurophysiology (described in Wyttenbach et al., 2014), neuronal cell culture, and *Drosophila* optogenetics. The mammalian neuron culture unit is described here.

Neurons are the fundamental units of the nervous system and like other cells they carry out myriad functions. Several functions, such as axon pathfinding and vesicular transport of neurotransmitters, are specific to neurons. Primary neuronal cultures from rodents are widely used to study basic physiological properties of neurons, and represent a useful tool to study the potential neurotoxicity of free radicals. Long-term culture of neuronal cells is challenging because mature neurons do not undergo cell division (Gordon et al., 2013). Researchers overcome this problem by establishing immortalized cell lines derived from neuronal tumors. While immortalized neuronal cultures have some advantages, primary neuron cultures are desirable for studies involving the toxic effects of reactive oxygen species.

Free radicals are generated endogenously by normal aerobic cellular metabolism and scavenged by cellular antioxidants. However, when antioxidant defenses break down or the brain is exposed to exogenous free radicals neurodegeneration occurs. For example, stroke induced ischemia may lead to oxidative stress from free radical attack on neurons, which contributes to neuro-degeneration. Free radical attack is implicated in Alzheimer's disease, Parkinson's disease, and other brain disorders (Uttara et al., 2009). Not surprisingly, antioxidants are increasingly used as therapeutic agents to slow neuronal loss. Primary neuron cultures are model systems for understanding the interplay between reactive oxygen species and the protective effects of antioxidants.

Prior to the beginning of the cell culture unit students are given key papers describing how cell cultures are used to study cellular processes in neurons. Students are asked to break those papers down into a series of "talking points" that include the research question being addressed, hypotheses being tested, experimental design, key methods required, major findings, and "next steps." The

idea is to build a working vocabulary and toolbox of methods that students can use in the four-week unit. The first three weeks are devoted to practicing the methods in their "toolbox," discussing the research papers, and designing independent experiments to be performed in the last two weeks. For example, week one is spent learning sterile technique. In week two, students use immunocytochemistry to fluorescently label microtubules and actin in the neuronal cytoskeleton. In the third week, neurons are exposed to high concentrations of hydroxyl radicals (generated from hydrogen peroxide via the Fenton reaction). The last week of the unit is used for student-designed research projects. Here we discuss the methods used in each module, suggest topics for student to explore, and discuss some of the pros and cons of this modular approach.

MATERIALS AND METHODS

Materials list

1-3 vials of primary rat cortical neurons (*BrainBits*®)
    -each vial contains 1 million primary neurons (sufficient for 2-3 teams)
250ml NbActiv1 culture medium (*BrainBits*®)
Silanized glass pipette with rubber bulb (*BrainBits*®)
Poly-d-lysine solution (50ug/ml, Sigma P6407)
4-well or 8-well culture dishes (LabTek or *ibidi*)
30^{o}C waterbath
Sterile 15ml plastic centrifuge tubes with screw cap
Centrifuge for 15ml plastic tubes
Automated cell counter (or hemacytometer slide)
Sterile hood containing:
    Adjustable volume pipettes
    Pipette tips (sterile)
    Trypan Blue (Sigma T8154) in microfuge tube
    Spray bottle with 70% ethanol
    Container for used pipette tips
    Beaker for waste disposal
37^{o}C, 5% CO_2 incubator
Fluorescence microscope (inverted preferred) with appropriate filter sets
Digital microscope camera (optional)
Antibiotic solution (optional)
Sterile gloves (varous sized)
Image J software (optional)

Dissociated primary neurons are purchased from BrainBit® LLC (http://www.brainbitsllc.com). They supply embryonic rat or mouse neurons from either the cortex or the hippocampus in vials containing approximately 1 million neurons. If proper aseptic techinque is followed, these neurons can last several weeks in culture. Purchasing ready to use dissociated neurons eliminates the need for expensive mouse breeding facilities and most Institutional Animal Care and Use Committee protocol approvals.

Module 1 Neuronal Cell Culture

Preparations

Maintenance of neurons in long-term culture requires strict adherence to aseptic technique to avoid contamination and potential loss of valuable cells (detailed descriptions can be found in Cote, 2001; Freshney, 2005). A day or two prior to the first lab, instructors should prepare the glass surfaces of the culture dishes using poly-d-lysine (Sigma P6407). There are several choices of suitable substrates including 4-well or 8-well chamber slides or 4-well µ-slide by ibidi ™ (Figure 1). The advantage of using 4-well slides over coverslips in a petri dish is that they reduce the volume of NbActiv1 media required and provide students with replicate experiments on the same microscope slide.

Lab tek 4 chamber surface area = 1.8 cm^2 per well
Ibidi 4-well slides surface area = 2.2 cm^2 per well

In a sterile hood quickly rinse each culture dish in 70% ethanol and then rinse by dipping in two changes of ddH_2O. Cover the dishes and allow to air dry. Add poly-d-lysine as 50 µg/ml to cover the glass surfaces of each culture well and allow to sit at room temperature for several hours. Aspirate off the poly-d-lysine (save for reuse) and rinse each well with ddH_2O. Cover culture dishes and allow to air dry. Aliquots of 20 µl of Trypan Blue (Sigma T8154) into 0.5ml tubes are also prepared ahead of time (one for each team of students).

Culture procedure

It is useful to have students practice aseptic technique prior to working with the neurons. This is acomplished by setting up the hood (Figure 1) with everything they will need, except a vial of water is substituted for the neurons. They go through the entire procedure decribed below to practice aseptic technique, before being given vials of dissociated neurons.

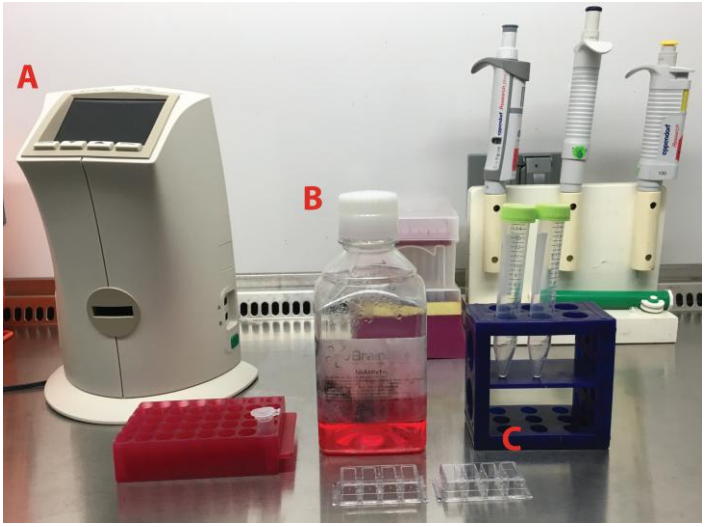

Figure 1. Typical sterile hood set up includes an automated cell counter *(A)*, a series of pipettes and sterile tips, microfuge tube containing trypan blue, NbActiv1 culture media *(B)*, sterile 15 ml centrifuge tubes, and several LabTek 4-well culture chambers *(C)*.

Remove the 2ml tube containing the dissociated primary neurons from the refrigerator (although *BrainBits*® neurons are best if used within 24 hours, they can be stored for several days at 4^{o}C). Remove carefully – DO NOT SHAKE. Warm the tube containing the neurons in a

30°C waterbath for 60 sec. Do not imerse the cap – just the bottom of the vial.

In a sterile hood transfer the contents of the entire tube of neurons to a sterile 15ml centrifuge tube using a sterile, silanized pasture pipette (also available from *BrainBits*®). Aggressively titurate the cells 5 times to break up aggregations in the media (a milky white cloud of DNA may still be present). Spin the 15ml tube of neurons at 1100rpm (200 x G) for 1 minute. Return to the sterile hood and aspirate off the supernatant leaving approximatley 50µl of media and the pellet. Flick the bottom of the tube with a finger and resuspend the pellet in 1ml of room temperature NbActiv1 media.

Remove 20µl of the cell suspension and add it to the 20µl of trypan blue prepared previously (1:2 dilution). One member of the team counts the neurons using an automated cell counter (Figure 1) or use a hemacytometer, while the other students plate the cells. (note: it is important to plate the cells within about 15 minutes of warming the tube).

In the hood add approximately 6-8mls of NbActiv1 to the tube containing the neurons (the amount of media depends on the desired density of neurons and the number of wells used per team). Swirl the tube gently to mix and pipette 700µl into each well of the Lab-tek 4-well chamber. Adjust the volume if using a different culture chamber. Immediately incubate the culture dish in a 37°C, 5% CO_2, 9% O_2,95% humidity incubator.

Every three days change half of the culture media with fresh 37°C acclimated NbActiv1. Neurons should adhere to the substrate within hours and extend neurites (axons and dendrites) within four days (Figure 2).

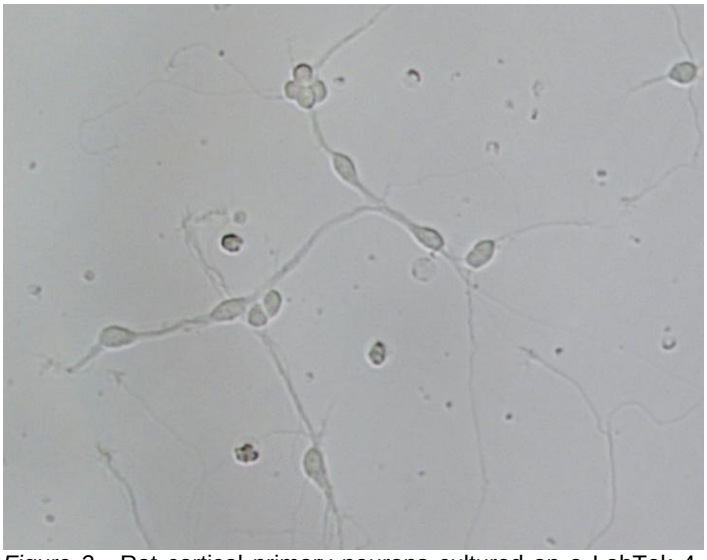

Figure 2. Rat cortical primary neurons cultured on a LabTek 4-well plate after four days incubation at 37°C, 5% CO_2, 9% O_2, 95% humidity.

Module 2 Cytoskeletal Immunocytochemistry

Immunocytochemistry is a powerful technique to observe the inner workings of cells. Neurons have elaborate cytoskeletons consisting of microfilaments, microtubules, actin, and other filamentous proteins. Neurite extension and pathfinding requires rapid reorganization of these cytoskeletal structures. Microtubules are also involved in intracellular protein trafficking along axons. All of these dynamic events are critical to proper neuronal funtion.

There are many techniques for labeling cytoskeletal elements (see www.thermofisher.com or www.cytoskeleton.com). Here we describe the use of fluoresently labeled antibodies to visualize tubulin and the phallotoxin phalloidin to tag actin filaments. Students are assigned readings covering basic immunofluorescent techniques prior to lab (Kumar and Rudbeck, 2009; www.dako.com/us/index/knowledgecenter.htm).

Staining procedure

Students first observe their cultured neurons under a microscope to check cell viability. They then aspirate off 80% of the culture media and add 3.7% paraformaldehyde fixative to each well for 30 minutes. Fixation stops cellular metabolism and crosslinks proteins.

The fixative is removed and the cultures are rinsed several times with phosphate buffer (PBS) over 5 minutes. The cells are permeabilized with 0.2% Triton X-100 in PBS for 10 minutes and rinsed several times in PBS. Staining is generally improved by blocking non-specific binding of the antibody with 5% normal goat serum in PBS for 10 minutes followed by several rinses in PBS.

A good primary antibody has high affinity and high specificity. We used 12G10 anti alpha-tubulin, a mouse monoclonal primary antibody (DSHB, Iowa City, IA), diluted 1:10. You should try a range of dilutions for optimum staining. If you are double staining with two different primary and secondary antibodies be sure the two primary antibodies come from two different host species (i.e., mouse and rabbit or mouse and goat). In most cases, one hour incubation with primary antibody at room temperature is sufficient. Rinse the cells with several washes of PBS to remove the primary antibody and add an appropriate secondary antibody to each well.

Blue	Green	Red
DAPI	AlexaFluor 488	AlexaFluor 568
AF350	AndyFluoro 488 GFP,Cy2, FITC	Cy3, Rhodamine Texas Red

Table 1. Common fluorophores for double or triple staining.

Secondary antibodies were conjugated to fluorophores (Table 1). We used a goat anti-mouse IgG AndyFluor 488 secondary antibody (GeneCopoeia) for the tubulin primary antibody. Secondary antibodies were diluted 1:100 -1:500 in PBS and incubated in the wells for one hour at room temperature. The neurons were washed 3-5 times in PBS to remove excess antibody. After rinsing off the secondary antibody we added Texas Red-X Phalloidin for 30 minutes to fluorescently label actin filaments. Actin staining is relatively simple because it does not require antibodies. Phalloidin belongs to a family of toxins isolated from the deadly “death cap” mushroom *(Amanita phalloides;* Lengsfeld et al., 1974*).* Labeled phalloidin conjugates are commonly used to selectively label F-actin in fixed cells. Finally, the cells were rinsed 3-5 times in PBS and viewed with an inverted fluorescent micrscope (Figure 3).

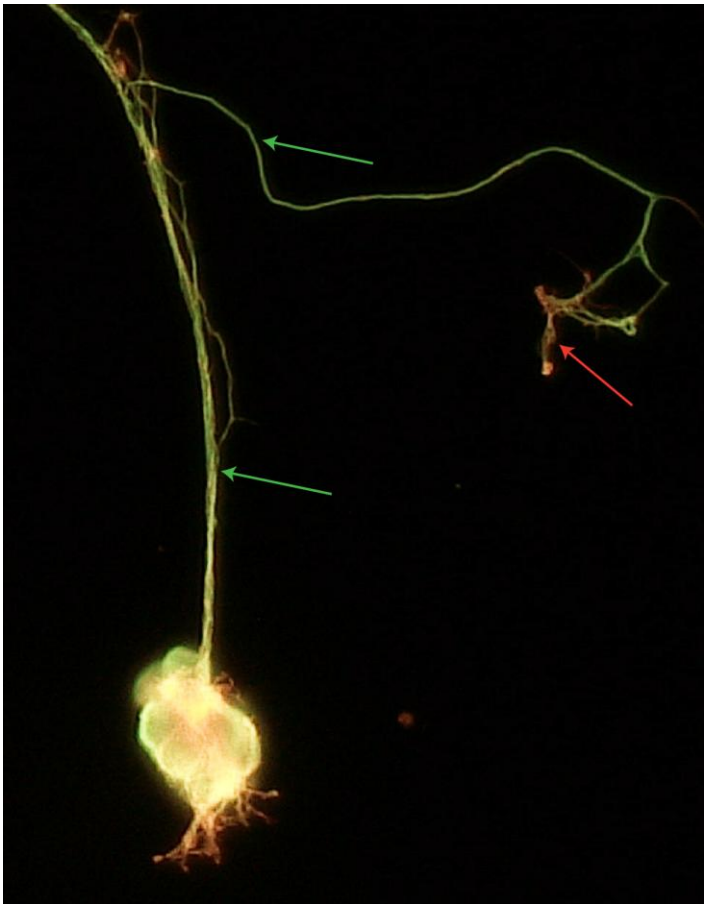

Figure 3. A group of neurons labeled with fluorescent AndyFluor 488 for tubulin (green arrows) and Texas red for actin filaments (red arrow).

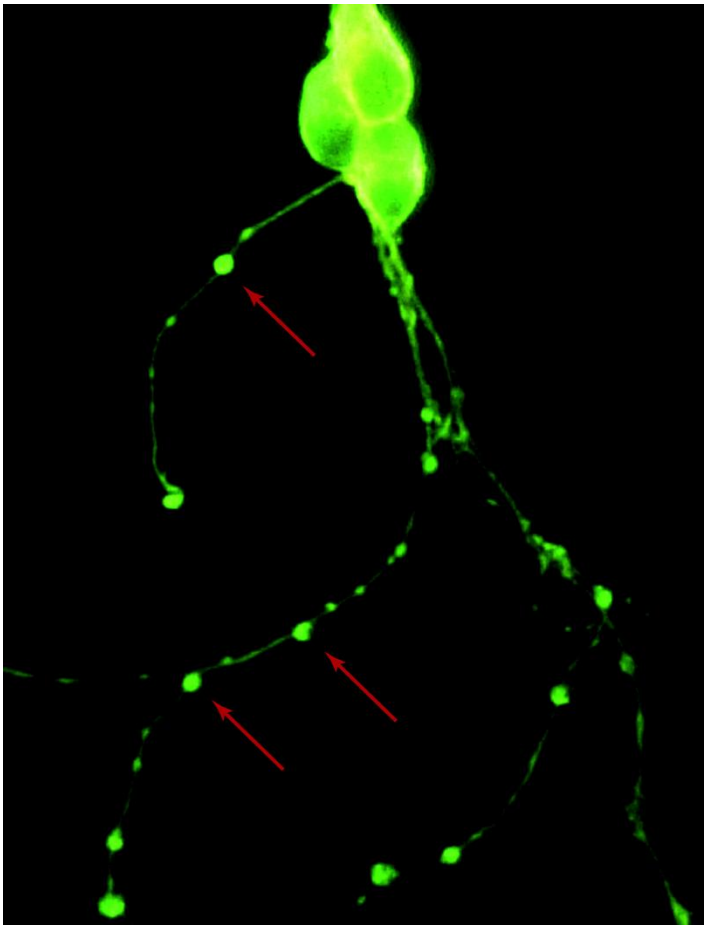

Figure 4. Three cortical neurons after exposure to H_2O_2 – generated free radicals for one hour. Arrows indicate areas of the neurites where blebs indicate membrane damage from lipid peroxidation.

The double staining procedure takes about three hours to complete. To save time, cells can be fixed and permeabilized prior to lab. Students should incorporate appropriate controls (i.e., wells lacking primary antibody) to check for non-specific binding (see Kumar and Rudbeck, 2009).

Labelled cells were photographed on a Zeiss Axiovert inverted microscope equipped with a 50W mercury light source, Zeiss 40X Neofluar lens, and Chroma 69000 Dapi/Fitc/Tritc filter set. Images were captured with a TrueChrome HD color camera or a Q-Imaging Retiga EXi camera. Students were encouraged to take a large number of digital photographs and transfer them to their course dropbox for use in their oral presentations.

Module 3 Free Radicals and Neurodegeneration

Several neurodegenerative diseases are associated with oxidative damage caused by excessive exposure of cells to reactive oxygen species such as free radicals. We use a model system of neurodegeneration by free radical attack to investigate the mechanisms by which reactive oxygen species damage neurons and how antioxidants provide protection against such damage. Specifically, we use a brief exposure of cultured cortical neurons to H_2O_2 to induce neuronal death. We assess cell damage morphologically by quantifying membrane blebs along neurites (Figure 4). Blebs are protrusions of the cell membrane caused by degredation of the underlying cytoskeleton (Charras, 2008; Fackler and Grosse 2008).

Cortical neurons are plated as described in module one but at a density of 7 x $10^4/cm^2$ ("low density"). Fresh 30% hydogen peroxide (H_2O_2) is diluted at various concentrations from 100µM to 100mM in culture medium (dilutions must be made within a few minutes of use). A small volume (100µL) of peroxide is added directly to each well with light agitation. Neurons are observed and photographed at 5 minute intervals for up to 1 hour. Images are imported into ImageJ for analysis (Abramoff et al., 2004; Schneider et al., 2012).

Module 4 Student Research Projects

The final module consists of student created experiments that build on the neuron culture techniques described in modules 1-3. Student experiments may include studies of:

- neurite growth rates in different growth media
- neurite pathfinding in response to growth factors/ inhibitors
- intracellular electrophysiology
- cytoskeletal degredation in response to free radicals
- the protective effects of various antioxidants

Students are very creative and there are literally hundreds of experiments to choose from. End of unit presentations are structured as short oral presentations in a format similar to those encountered at a scientific meeting (15-minute presentations with Prezi or PowerPoint slides).

DISCUSSION

Neuronal cell culture is a relatively simple preparation that allows students to investigate diverse cellular functions. Purchasing pre-dissected primary rat neurons eliminates the need for rodent colonies, IACUC protocols, and tedious embryonic dissections. Instead students can focus on learning sterile technique and immunocytochemistry. Plated neurons extend neurites and make synapses with adjacent neurons within 4-5 days at 37^{o}C. The multi-well culture dishes allow replicate experimental chambers that increase efficiency while reducing the volume of culture media, antibodies, and phalloidin. More importantly, neuronal cell culture allows students to explore the cellular and molecular basis of neuronal connections in a relatively simple and accessible system.

Our course consists of four modules over a 4-5 week period. The first week students practice sterile technique and plate rat cortical neurons in multi-well chambers. At the same time students are reading and discussing scientific papers that demonstrate how culture techniques are used to explore cellular function (for example, Vitrio and Zheng, 2012; Munnamalai and Suter, 2009). The next two modules use immunocytochemistry and neurotoxicity studies. Here students visualize the neuronal cytoskeleton using antibodies and explore how free radicals degrade the cytoskeleton and lipid membrane. The goal is to introduce two simple techniques that students can build upon as they design their own experiments in the final module.

In our experience, students are able to master these techniques with ease. Additionally, these techniques generally do not take the full lab period, leaving time for discussing scientific papers and planning the next module. Because they are modular, instructors may choose to eliminate or substitute one of the module 2 or 3 techniques to make more time for student-generated experiments (module 4).

The logistics of the four-week cell culture unit can be complicated. Typically students begin formulating ideas two weeks prior to module 4. They conduct literature searches and propose their research idea in a short 3-5 page “grant” proposal, which details the hypothesis and predictions, provides a list of materials, and describes how the data will be analyzed (i.e., what statistical tests or other measurements will be used). Each team’s grant proposal is “peer-reviewed” by two other student teams, revised, and submitted for approval by the instructor. Instructors must pay attention to the cost and shipping time of additional reagents or supplies required by the projects.

There are several areas where instructor attention is critical. The first is that some student projects may require additional time outside of the scheduled lab period to be completed. For example, neurite growth experiments may require recording images at specific intervals over the course of the day. At our institution, students are given access to labs outside of class time via keypads on the lab doors. Since many of our courses require students to return to lab outside of the scheduled time, students consider this a normal part of the lab experience. At institutions where this kind of access is not permitted, instructors must carefully limit certain types of projects.

A second area where students may have trouble is with sterile technique and the initial plating of neurons. To avoid problems, we have students practice sterile techniques using “fake” neurons at first. For maximum yield, students have only about 15 minutes from opening the vial containing the primary neurons (from BrainBits) to placing their plated neurons in the 37^{o}C incubator. Practicing with “fake” neurons beforehand builds confidence and allows them to perfect the timing of each step in the procedure. Although all student teams successfully cultured neurons, it may be advisable for instructors to plate a couple of extra dishes of neurons just prior to lab as a back up.

Students may also have trouble calculating and performing appropriate dilutions. Although instructors could provide all of the reagents in ready-to-use stocks, we prefer to have students prepare their own reagents. This requires spending some lab time reviewing calculations and dilution techniques. However, we believe this is time well spent because it reinforces the linkage between neuroscience and biochemistry. This provides students with a greater understanding of their laboratory exercises, as well as instilling a sense of ownership of their research projects. For the instructor it means extra vigilance in checking reagent preparation. We have students submit their calculations for approval ahead of time to avoid wasting expensive reagents.

Small population size and lack of a control population (students who did not have the cell culture unit) prevent formal assessment procedures. However, student self-reported assessment of the neuron culture unit indicates that they enjoy this unit the most. Students report that seeing a single neuron in culture extending its neurites to make connections with neighbors reinforces the concepts of pathfinding, synapse formation, and neurodegeneration discussed in the lecture part of the class. There is uniform praise for the student-generated projects as well. Comments like “I finally felt like a real neuroscientist” are common. Many students reported that the unit was challenging, but rewarding.

In sum, neuron culture is a relatively simple and readily accessible teaching unit for undergraduates. It is also commonly used in neuroscience research labs. Thus, students motivated to pursue graduate research may benefit from learning this experimental approach.

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Address correspondence to: Dr. James Ryan, Biology Department, Hobart and William Smith Colleges, Geneva, NY 14456 USA. Email: ryan@hws.edu