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Introducing immunohistochemistry to the molecular biology laboratory

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Abstract

Widely used in research laboratories, immunohistochemistry (IHC) is a transferable skill that prepares undergraduate students for a variety of careers in the biomedical field. We have developed an inquiry-based learning IHC laboratory exercise, which introduces students to the theory, procedure, and data interpretation of antibody staining. Students are tasked with performing IHC using an "unknown" antibody and then asked to identify the cells or molecular structures within the nervous systems specific for that unknown antibody. In two lab sessions, students are exposed to handling of delicate brain slices, fluorescent microscopy, and data analysis using the Allen Brain Atlas (ABA), an online freely accessible database of mRNA transcript expression patterns in the brain. Here, we present guidelines for easy implementation in the classroom and assess learning gains achieved by the students upon completion of the IHC laboratory module. Students clearly displayed an increase in knowledge in data interpretation, procedural knowledge, and theory surrounding IHC. Thus, this module works as an inquiry-based learning based method to introduce IHC principles to undergraduate students.

KEYWORDS

active learning, fluorescent microscopy, immunohistochemistry

1 | INTRODUCTION

Undergraduate biology educators are tasked with the challenge of designing engaging lab exercises that are not just relevant to their curriculum, but also teach deployable skills for the next generation of scientists. It is especially difficult to design such labs under a tight budget when studying biological tissue to the degree of detail that cellular and molecular biology demands. In addition, labs must be efficient given the limited lab time allotted for undergraduate programs.[1] To accommodate these restrictions, we have developed an active

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learning immunohistochemistry (IHC) laboratory exercise.

IHC staining is a transferable skill that is popularly used in the diagnosis and classification of cancerous tumors, localization of molecular biomarkers, and the identification of a multitude of expressed proteins in biological tissue. Due to its vast applicability to fields such as disease informatics and drug development, IHC is a common research tool in modern protein chemistry and pathology laboratories.[2] Here, we describe an active learning experiment where students engage in immunostaining on mouse brain slices. Active learning encompasses many pedagogies, but we focus on collaborative and inquiry-based learning in this laboratory experiment. Students demonstrate inquiry-based and collaborative learning by first visualizing unknown antibodies under a microscope, while analyzing mRNA transcripts from the Allen Brain Atlas (ABA). Students then use their observations of the antibody staining and images from the ABA to form hypotheses as to what cellular or molecular structure the antibody detects. Thus, this study encourages students to engage in open-ended investigation led by their own questions. The ABA shows a three-dimensional atlas of gene expression in the brain of an adult mouse, allowing researchers to corroborate gene expression patterns in different species using methods such as IHC.[3] Applicable to this study, the ABA enhances undergraduate education by serving as an easily accessible resource and guide to study the brain's structural organization and promote in silico laboratory exercises.[3] In this laboratory exercise, students are encouraged to engage in inquiry-based investigation, conduct analyses using antibody staining images, and interact with the brain's molecular components.[3]

Active learning serves as an effective learning strategy in undergraduate classrooms.[4,5] This teaching lab serves to build upon previous IHC labs that often contain an active learning subtype called collaborative learning.[2,6,7] Collaborative learning is also displayed in this lab by encouraging students to work in small groups to navigate the exercises and communicate findings in an oral presentation. According to one study, collaborative learning has improved several learning outcomes compared to solitary work.[8] Not only do students show improvement in academic achievement and retention of the material, but they also experience a boost in self-esteem and attitude.[4,8]

In addition to collaborative learning, this teaching laboratory adds a further element of active learning called inquiry-based learning. Previous research of inquiry-based learning has demonstrated the enhancement of students' acquisition of critical scientific-inquiry skills, understanding of course content, and knowledge of scientific processes and literacy.[9,10] Inquiry-based learning is enforced by exposing IHC to the students in which they are blinded to both the antibody and protein. By making the student a blind participant to the specifics of both the antibody and antigen, students are forced to practice inquiry-based learning in a collaborative manner. Thus, this molecular biology teaching laboratory will expose students to an active learning laboratory through the use of both a collaborative and inquiry-based format, allowing them to use critical thinking to effectively interpret data, communicate findings to others, and summarize results as a class. Specifically, the student learning goals for the laboratory exercise are: (1) To acquire conceptual knowledge of utility and applications of IHC and confocal imaging, (2) To practice IHC techniques with mammalian brain slices, (3) To compare images of collected antibody staining with ABA images to determine whether an antibody provides specific staining. During the first 3-hours session, students follow a speed IHC protocol for brain slices in culture wells, investigate the ABA, and mount samples on microscope slides in an undergraduate-friendly protocol. In a subsequent session, students image samples on a fluorescence microscope and present their findings to the class. The module can be completed in two 3-hours sessions. The proposed lab helps students learn a transferrable laboratory technique and gain insight into protein expression and the anatomical structure of the brain. Students gain a valuable lesson in the cell biology of the nervous system, while also enhancing their "research" experience with useful, interactive online tools. Students are introduced to the ABA, a free online database to examine *mRNA* expression in specific brain regions.[3] These exercises offer a rare opportunity for students to interact with the brain and visualize neurons and glia composing the brain.

2 | MATERIALS AND METHODS

2.1 | Classroom materials

Prior to the lab session with students, mouse brain slices were prepared. All procedures were approved by the IACUC of the W.M. Keck Science Department and UC Irvine. Institutions without access to laboratory mice can order perfused whole mouse brains from BrainBits LLC (www.brainbitsllc.com) with equal success. The 40 μm brain slices were prepared using a cryostat and stored in 0.1 M PBS + 0.1% sodium azide. Sodium azide acts as a chemical preservative and allows long-term storage of brain tissue, so that a single mouse brain can provide tissue for one academic year. A full list of materials, including catalog numbers and pricing, is available in the Supplementary Materials (Supplemental Table 1).

2.2 | Classroom procedure

Upper-division undergraduate students work in groups with 2–3 members to investigate their unknown antibody. Each group is provided two 40 μm brain slices suspended in 0.1 M PBS and stored in separate wells in a 24-well culture plate. For storage beyond 24 hrs, the edges of the culture plate should be wrapped with parafilm to prevent dehydration. For the full list of reagents prepared for each group, see faculty instructions provided in Supplement 2. One brain slice is reserved as a negative control, which receives identical treatment as the experimental condition but lacks the primary antibody tested in the experimental condition. If one has enough tissue, further negative controls could also include tissue that lacks a secondary antibody. Students use micropipetters to prepare the primary antibody solution, primary control solution, and secondary antibody solution. To speed the incubation process, primary antibodies are used at 1:500 concentration for 45 min at room temperature. Brain slices are washed by removing solutions in culture wells with a transfer pipette and adding new 0.1 M PBS solution using a squeeze bottle. Secondary antibodies are used at 1:250 concentration for 30–45 min at room temperature. Following secondary antibody incubation, brain slices are again washed before mounting on microscope slides. For full details on washing, incubation and mounting procedures, see protocol provided in Supplement 3.

2.3 | Transferring brain slices

Standard soft bristle paint brushes are used to move brain slices from culture wells. Paintbrushes are gently moved underneath brain slices to lift tissue from one body of PBS to another.[11]

2.4 | Mounting brain slices

To mount brain slices, students fill a large petri dish with 0.1 M PBS. A microscope slide is placed at the bottom of the petri dish and a paintbrush is used to move a single brain slice from the culture well to the petri dish of PBS. As the paintbrush is used to stabilize the brain slice, the microscope slide is used to gently lift the brain slice out of the PBS. This step can be repeated if the brain slice is not mounted flat in a single layer. Excess PBS is removed from the microscope slide using a Kimwipe, avoiding contact with the brain tissue. The sample is allowed to air dry until there is no longer a bead of liquid above the brain tissue. Tissues are then mounted with Vectashield mounting solution to allow for overnight DAPI staining. DAPI is commonly used as a nuclear marker due to its high specificity and fluorescence quality, which allows it to be used to identify DNA and thus nuclei.[12] Students should be reminded that only a single drop of Vectashield mounting solution is needed to mount the tissue. Samples for the negative control and the experimental condition are mounted on separate slides for ease. Prepared coverslips can be imaged as soon as 30 min after mounting, but are typically imaged 1–2 weeks after the first 3-hour class period in the standard lab exercise run at The Claremont Colleges and the University of California, Irvine. Microscope slides should be stored in the dark at 4°C.

2.5 | Visualization

In the second lab session, brain slices were imaged on a shared departmental or core facility confocal microscope. Images of both the stained and control sections were collected by student groups, which were then used for short presentations. Each group received a 20–30 min appointment with the confocal microscope. During this appointment, students were introduced to the x-, y- and z-dimensions of the tissue and received guidance on how to interpret the green and blue staining in their images.

If time permits, the instructor can arrange individual meeting times with student groups to give students more access to imaging and exposure to confocal microscopy. This gives students the opportunity to learn more details of optimizing image capture and helps 'demystify' the use of fluorescence microscopy. Visualization can also be done on most fluorescence microscopes if a confocal is not available.

2.6 | Group presentations

Confocal microscopy resources are often limited for teaching laboratories at the undergraduate level. Thus, when one group of students was active on the confocal microscope, the remaining groups were asked to create a 3–5 min google slide oral presentation about their work. To aid the students with their oral presentation, the students were provided "thought questions" (Supplement 3). These "thought questions" are arranged in a specific order to reflect on the learning outcomes and goals stated earlier. At the end of the second laboratory session, students presented their findings in front of the class. Questions from other groups were encouraged to promote a deeper understanding of the laboratory exercise.

2.7 | Assessment

Students' prior exposures to forms of classroom laboratory exercises were estimated using self-assessment on a

5-point Likert scale. In order to assess student attainment of IHC concepts, procedural knowledge and data interpretation, an 8-question pre/posttest was administered before and after the IHC module. Multiple-choice questions included three "circle all that apply" problems. For these questions, answers that did not include all correct responses were scored as incorrect.

2.8 | Participants for assessment

Students from the W.M. Keck Science Department shared by Claremont McKenna College, Pitzer College, and Scripps College were recruited for the study (n = 15). The study was considered exempt by the Keck Science Department Institutional Review Board.

3 | RESULTS

Students compared experimental conditions, which were probed with a primary antibody, with negative control conditions that were processed similarly but not probed with a primary antibody. A primary antibody was considered specific to the antigen if students found specific staining in experimental conditions that matched mRNA staining patterns found in the ABA and protein patterns published in literature, if applicable, and did not see this pattern in the negative control condition. Students presented their findings in an oral presentation. All students obtained successful DAPI staining of the nucleus and evidence to determine whether their assigned primary antibody was specific to the marketed antigen (Figure 1). In all cases, evidence included fluorescent images from control and experimental conditions collected at the same image acquisition settings and a summary of expected expression patterns based on data available from the ABA. Student groups varied in whether they presented image stacks, single snapshot images, several snapshot images, or image projection files prepared using imaging software that accompanied the classroom microscope. Students also varied in how they presented the information that they gleaned from the ABA, ranging from summary histograms and text descriptions to raw images. In some cases, students also included images of expected expression patterns from peer-reviewed primary literature. By comparing their antibody staining to the negative control, students determined whether there was any puncta pattern beyond non-specific staining. Comparisons of student-generated antibody staining data to expected expression patterns further provided evidence on whether the candidate antibody provided specific staining. Refer to Supplemental Materials for list of confirmed antibodies and purchasing information. Confirmed antibodies can be used as positive controls.

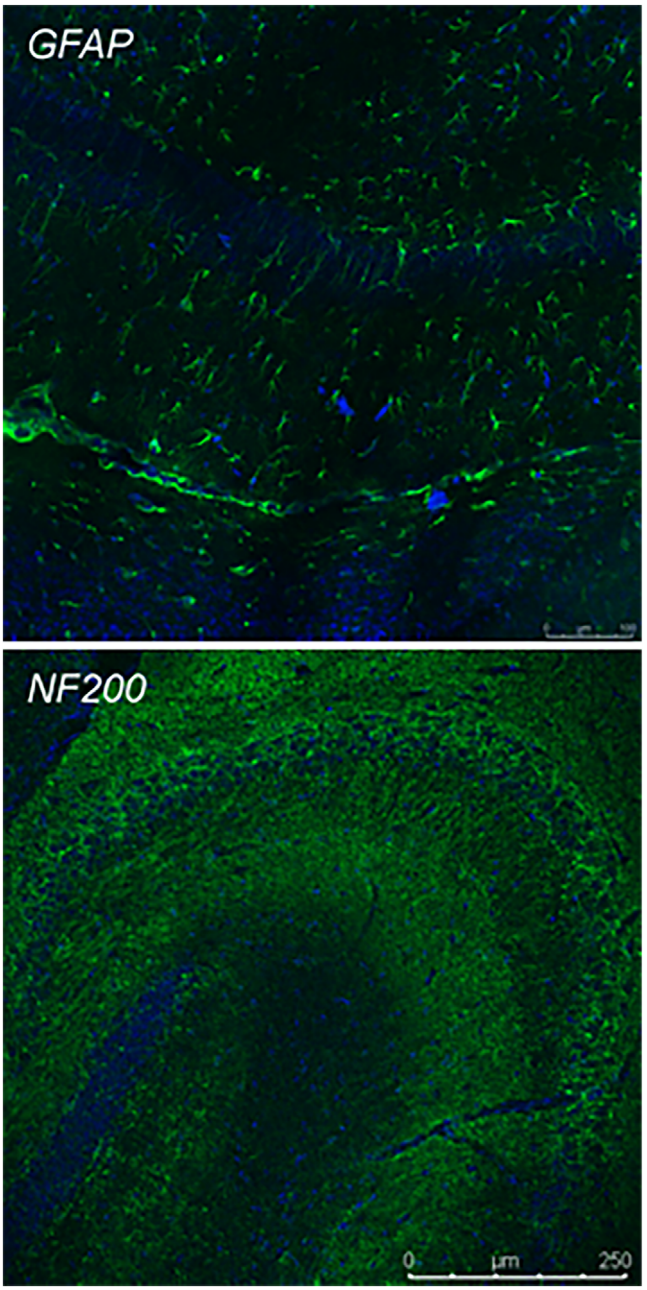

FIGURE 1 Sample student images. Glial fibrillary acidic protein (top) and neurofilament-200 protein (bottom) staining in green, overlaid with blue DAPI staining. Scale bars: 100 µm

3.1 | Student experience

Prior to the introduction of the IHC lab exercise, the students' past exposure to forms of classroom laboratory exercises was assessed. Assessment of student experience focused on student report of experience prior to the lab

exercise and perceived experience following the lab exercise. The 87% of students reported having 'much' or 'extensive' experience in performing a scripted lab or project in which they knew the expected outcome (Figure 2). In contrast, none reported having 'extensive' experience in performing a lab or project where the outcome was unknown.

The designed IHC lab lesson gave students exposure to a lab project where no one knew whether the antibody would show specific staining. In post-tests, the majority (81%) of students now reported having at least 'some' experience with projects where no one knew the outcome (Figure 2). The lab lesson also encouraged engagement with primary scientific literature, and all (100%) students reported having at least 'some' experience reading primary scientific literature in post-tests. As expected, post-tests revealed that students perceived gained experience in communication of scientific discoveries, with 19% reporting 'extensive' experience and 38% reporting 'much' experience in post-tests compared to 0% and 20%, respectively, in pre-tests.

We also compared student reports of their current level of ability in relevant laboratory skills before and after the IHC lab exercise. Prior to the IHC lab exercise, 60% indicated they had no experience or felt inexperienced with IHC, 73% reported this of the ABA and 47% in handling brain slices (Figure 2). After the IHC lab exercise, the majority of students now reported they had at least a 'little' experience in these three areas (Figure 2). Thus, the lab exercise improved exposure to a widely used lab technique, though 6%–7% still reported that they had no experience or felt inexperienced.

Student self-reports of experience level provided an estimate of student background and perceived gains in laboratory competencies. Instructor assessment focused on assessing student ability to acquire conceptual knowledge of the utility and applications of IHC.

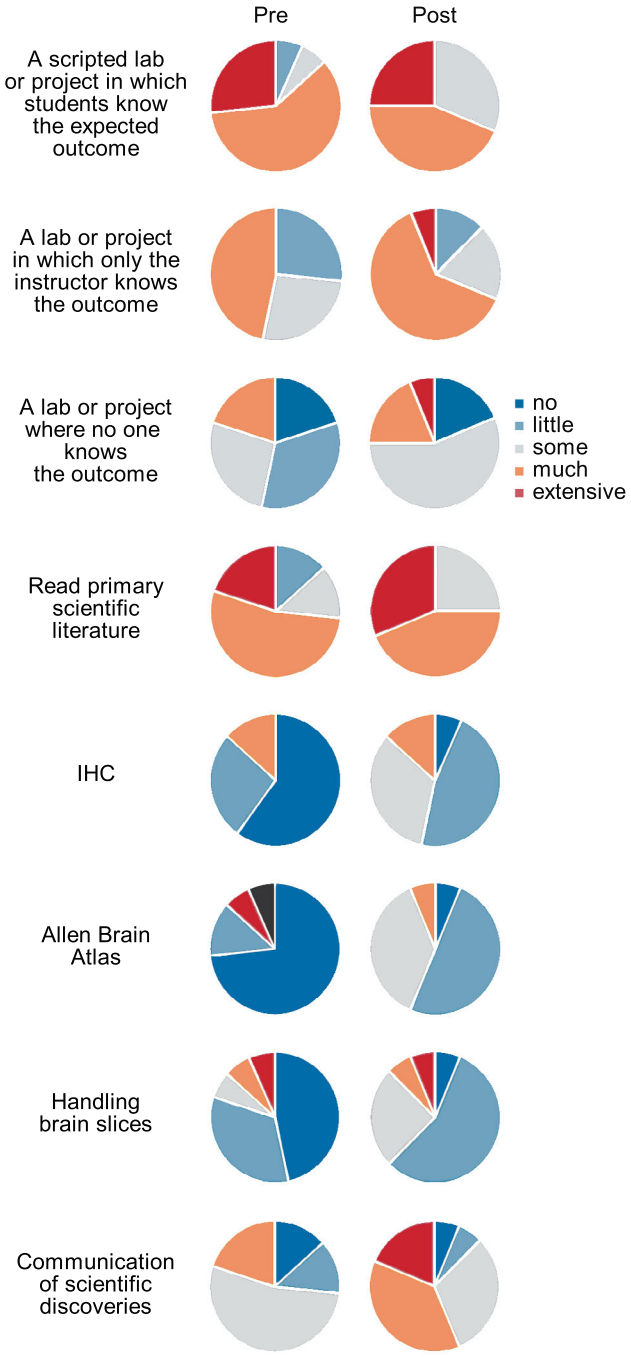

FIGURE 2 Increased exposure to transferrable lab skills. Students reported on experience with scripted, blinded (study in which only the instructor knows the expected outcome), and active learning lab experiences and specific lab skills before and after IHC lab lesson. Warm colors indicate greater experience or mastery of the element, while cooler colors indicate minimal exposure and experience. A shift to warmer colors indicates increased experience with the indicated element. Black chart regions indicate students preferred not to answer

3.2 | Learning of theoretic framework

Students demonstrated that they had adopted a biological inquiry mindset through oral presentations. Presentations showed that students grasped the context surrounding their research question, articulated testable research question(s), pursued experiments to test their identified research question, and generated tentative conclusions given the limitations of their work. In order to further assess skills in inquiry and student learning of the theoretical framework underlying IHC, we tested students using a multiple-choice, choose-all-that-apply questionnaire. Questions tested whether students accurately identified research questions in which IHC could be utilized and the rationale behind the use of a secondary antibody. Prior to the laboratory exercise, students correctly answered, on average, 7% of these questions (Figure 3). When this assessment was given again after the

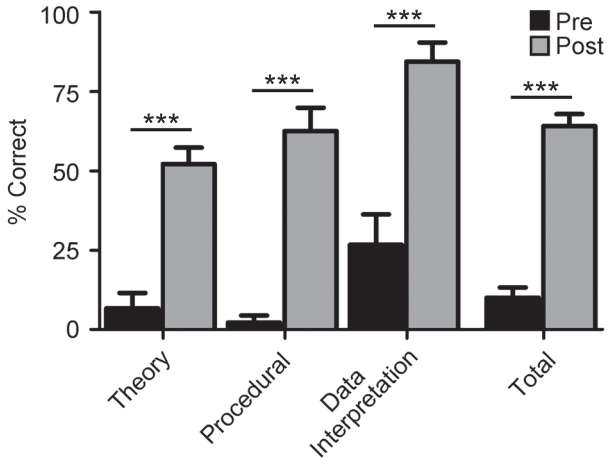

FIGURE 3 Learning gains assessed by comparison of pre- and post-tests. Students demonstrated better understanding of the theory underlying IHC, procedural knowledge of IHC and interpretation of potential experimental outcomes. ***$p < 0.0001$ for pairwise comparison of pre and postscores using Student's *t* test

laboratory exercise, students showed a statistically significant increase in the percent of correct answers, demonstrating that an inquiry-based learning exercise does have an impact on the ability of students to learn the concepts behind a procedure (Figure 3). Students demonstrated that IHC allowed inquiries that explored the location of a protein of interest and whether a cell or tissue expressed the protein of interest as opposed to inquiries on RNA expression. Similarly, to assess student learning of procedural knowledge of IHC, students were asked to identify the materials needed for IHC and the time required for the procedure. Students answered, on average, 2% of questions correctly in pre-tests compared to 62% of questions in post-tests. The multiple-choice, choose-all-that-apply assessment also tested data interpretation when students were given scenarios of hypothetical data and asked to interpret the results. Question prompts described staining strength and puncta appearances in the negative control and experimental conditions. To answer correctly, students needed to understand how procedural mistakes affected image quality and identify generic differences between negative control and experimental conditions. Assessment questions used to assess student learning gains can be found in the supplemental material (Supplement 4). Students showed a three-fold increase in their ability to accurately interpret IHC data. Students also demonstrated an awareness of how the ABA can be used in their inquiry. Students benefited from the IHC laboratory module in all three aspects (*t* test, $p < 0.0001$). While these results were not a surprise, subjective quantitative analysis allowed the authors to ensure inquiry-based learning had occurred.

4 | DISCUSSION

In this study, our aim was to equip upper-division undergraduate students with IHC skills in an inquiry-based learning laboratory exercise. Active learning laboratory exercises have been shown to improve student learning, retention, scientific literacy, and communication skills.[13] Students were introduced to an IHC-based laboratory exercise in which students are able to handle mouse brain sections, stain these sections with various antibodies, and process the data that they generate. Pre-tests showed that students were not commonly introduced to antibody staining or laboratory experiments in which the answer is unknown; however, post-tests indicate that this exercise successfully enhanced students' understanding of laboratory techniques such as identifying cellular or molecular structures detected by the antibody using IHC and ABA. This laboratory exercise familiarized students with an IHC protocol, including evaluating the specificity of an antibody. By comparing generated IHC data with known mRNA expression patterns found on the ABA, students were able to form hypotheses and draw conclusions as to which peptide or protein shows specificity to the antibody. We show an active learning teaching laboratory that focuses on collaborative and inquiry-based learning positively impacts student abilities in conceptual knowledge, technical skills and data interpretation. In self-assessments, students report greater levels of laboratory experience for handling mouse brain slices and performing an IHC experiment. Students also show greater understanding of navigating the ABA, reading primary scientific literature, and communicating their discoveries to others.

The described IHC laboratory exercise also exposed students to the ABA with the secondary aim to improve student ability to navigate a freely accessible online database. With increasing amounts of big data and newly developed infrastructure to access and share the information, the number of online tools available has increased and there is a pressing need to train students in information literacy.[14,15] An information-literate individual is able to determine the extent of information required to answer their question, effectively access and evaluate this information, and properly extract the necessary information for applications.[16] Widely used, the ABA offers scientists a comprehensive digital map detailing more than 20,000 gene expression patterns in the mouse brain.[3,17] It is important for students to acquire practice navigating these types of tools, which enhance their understanding of common laboratory techniques and procedures.[18] A significant amount of time has been put into creating digital curricula and big data resources like the ABA; however, these digital libraries have been underutilized with instructional resources only beginning to be introduced.[19,20]

This laboratory exercise also improved student engagement with primary scientific literature. Faculty often indicate that students training to be future scientists must develop scientific writing skills and a familiarity with journal articles; however, students often lack this experience.[20] Exposure to primary literature has been shown to increase student scientific and information literacy.[21] Furthermore, it has been shown that greater student information literacy improves student confidence when reading primary literature and navigating discipline-specific online tools.[21] Information literacy is an important skill in the biomedical field, since the ability to identify a problem and then acquire the necessary information to modify one's research are key aspects to becoming a successful scientist.

At the University of California, Irvine, the neurobiology laboratory meeting times are confined to 3 h. To shorten class times, students learn didactic information about IHC outside of class time, through a laboratory manual and interactive Rocketmix online modules, which introduce and assess acquisition of content and procedural knowledge. The Rocketmix module includes annotated videos of the more complex handling procedures, allowing students to have a front-row view of the procedure repeated before arriving in the classroom. Instructors preassign student groups by distributing student strengths and check that students' antibody calculations are complete before the start of class. The 15 min in a prior class session were also used to introduce students to pipetting 1 μL into larger volumes. Faculty instructions on how to aliquot reagents to enhance speed while minimizing waste can be found in Supplement 2. The IHC exercise may be used to emulate a real research project over the course of 4 weeks, where students use the validated antibody to compare protein abundance in two different mice. In this extension, students are also introduced to the Western blot. Both techniques use the same primary antibody; however, the techniques utilize different secondary antibodies. In IHC, students use an Alexa488-tagged secondary antibody to visualize the spatial localization of the fluorescently tagged secondary antibody in a murine brain slice. In a western blot, students use a HRP-conjugated secondary antibody to quantify proteins from a murine brain homogenate. Procedurally, during the week following fluorescent microscopy, students use SDS-PAGE (**s**odium **d**odecyl sulfate-**p**oly**a**crylamide gel **e**lectrophoresis) to separate proteins by molecular size. In the fourth week, students perform a western blot to compare the abundance of the protein in two different mice followed by a colorimetric detection system. This adaptation requires more class time and institutional resources. For institutions that do not wish to invest in animal husbandry, the IHC laboratory exercise has also been implemented successfully with vendor-provided perfused mouse brains. Ordering information can be found in Supplement 1. Use of perfused mouse brains does not require approval from an Institutional Animal Care and Use Committee (IACUC), thereby lowering the barriers to implement the IHC laboratory exercise.

Although the IHC exercise is designed to be undergraduate-friendly and simple to implement into the laboratory, students may experience challenges that require guided practice. Mounting tissue onto slides is a crucial step to ensure proper viewing of the specimen and is most efficient with prior experience, so students may benefit from practicing with sample tissue before they attempt the IHC protocol.[11] Confocal fluorescence microscopy is not commonly taught at the undergraduate level, so students are encouraged to work with a trained technician to learn the basics of microscopy and improve image quality before visualizing the antibodies. Learning how to properly use a pipette is a necessary skill in the lab, since poor accuracy using pipettes is a common laboratory error, especially given small quantities. Students should be instructed with the proper technique, and then begin practice pipetting with water several times before attempting the IHC exercise.

Due to the versatility of this lab, future experiments can be enhanced by modifying certain parts of the experiment. For instance, primary antibodies that are already conjugated to a fluorophore can be supplemented. Although our laboratory exercise serves as an affordable model for undergraduate students to familiarize themselves with IHC, this adjustment can be more costly but allows for identification of the target protein without need for a secondary antibody.[22] In addition, multiple primary and secondary antibodies can be used to label multiple cell types at once without increasing the completion time. Using a secondary antibody aids in identification and sorting of the antigen, since the secondary antibody binds directly to the primary antibody.[23,24]

With the modernization of STEM (science, technology, engineering, and math) education, there has been an increasing number of calls to improve undergraduate teaching in STEM fields. These calls emphasize a fundamental shift in undergraduate education from an instructor-centered approach to student-centered education.[5,25] Here, we have shown that the implementation of an inquiry-based learning IHC exercise into an undergraduate molecular biology laboratory course improves a number of student learning outcomes. Students reported that they had gained more experience with inquiry-based and collaborative learning projects and the use of a practical laboratory technique. Students also showed greater understanding of the theory behind IHC, as well as their ability to analyze expression patterns on immunostained brain sections. With the use of this laboratory exercise, students gain first-hand experience handling and staining

tissue sections and interpreting the results. This laboratory exercise is simple and easy to implement into an undergraduate lab, yet provides students with a transferable skill set that is commonly and diversely used in biomedical research.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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