RSGC
Royal St. George's College

The Young Researcher

2025 Volume 9 | Issue 1

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Shriya Singh

Recommended Citation
Singh, S. (2025). Optimizing antibody concentrations for immunohistochemistry in neurotrauma research. *The Young Researcher,* 9(1), 98-133.
http://www.theyoungresearcher.com/papers/singh.pdf

ISSN: 2560-9815 (Print) 2560-9823 (Online) Journal homepage: http://www.theyoungresearcher.com

Optimizing Antibody Concentrations for Immunohistochemistry in Neurotrauma Research

Shriya Singh

Abstract:
Objective/research question: What primary and secondary antibody concentrations optimize the visualization of immunohistochemical (IHC) stains for antigens prevalent in neurotrauma research?
Method: IHC was conducted on six different antigens, with one control. A scoring guide was used to objectively determine which IHC stain was visually optimized. Tested concentrations were chosen based on the manufacturer-recommended concentration and concentrations used in past papers.

Results: The antigens and their respective optimized concentrations are as follows: IBA1, P500S250; GFAP, P1000S500; MAP2, P1000S500; AQP4, P50S500; CD83, P50S500; MBP, P1000S250.
Conclusions: Results suggest a gap in the current secondary antibody literature. Future research should use this study as a reference to facilitate neurotrauma research, limit scientific experimentation on animals, and improve data collection. The goal is to ultimately improve TBI pathology comprehension and find therapeutic treatments to ameliorate patient outcomes.

Keywords: Microglia, astrocytes, traumatic brain injury, immunohistochemistry, antibodies, optimization, staining, neuroinflammation, neurotrauma.

Introduction and background information

Traumatic brain injury (TBI), a result of mechanical forces being applied to the head, displacing the brain within the skull, and disrupting neurological function throughout the brain, affects around 10 million people worldwide.[1-2] Despite the substantial amount of literature on the cellular processes of TBI, the mechanisms that constitute its pathophysiology still need to be further researched.[1] Specifically, understanding the role of acute and chronic neuroinflammation is essential for comprehending how TBI advances over time and how treatments need to be tailored to this progression.

Neuroinflammation involves glial cells, non-neuronal cells in the central nervous system (CNS). Microglia and astrocytes–immune cells specific to the brain–are two of the most researched glial cells in TBI. To study these cells, researchers often use animal models and investigate the prevalence of these cells over time after impact. They inflict a force onto these animals, obtain a tissue sample from their brains, conduct analyses to determine concentrations of glial cells and maps of neural circuits, and repeat these steps.[3] Midline fluid percussion brain injury is one example of an experimental model of diffuse TBI

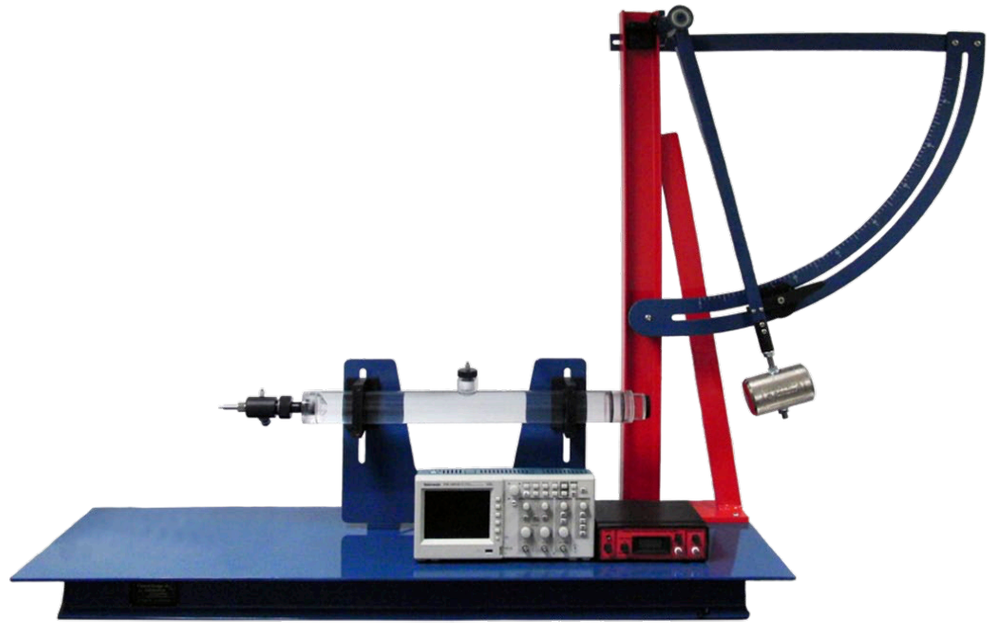

Figure 1. Image of the midline fluid percussion brain injury device.[4]

where a controlled impact of fluid is delivered into the midline of the skulls of rats and mice.4

Injury is induced through a 20-millisecond fluid pulse delivered into the brain through a craniectomy–a surgical procedure that removes part of the skull–and a surgically implanted injury hub. The fluid pulse is generated by the pressure wave created when the weighted end of the pendulum (right) hits the fluid cylinder (middle).[4]

Researchers may conduct analyses at different times after the force is inflicted; in fact, some researchers wait for a specific number of days post injury (DPI) for the onset of specific pathology (e.g., waiting 7 DPI for microglia studies). In some studies' research protocols, animal models perform behavior tasks to better elucidate the relationship between brain trauma and physical actions.

Immunohistochemistry (IHC) is a widely used technique in tissue analysis that uses antibodies–proteins that bind to specific markers on cells–to visualize various components within tissue samples. These markers are called antigens. By using antibodies specific to certain antigens—whether on the surface of cells or within tissues—and attaching a visibility agent to these antibodies, researchers can obtain stains to visualize the location and abundance of specific proteins or cell types. However, optimizing the visualization–that is, maximizing the clarity and usability–of tissue samples remains a challenge, particularly when using new antibodies. Researchers often lack standardized antibody concentrations, leading to trial-and-error testing on non-experimental tissue, which results in unnecessary waste of animal samples. Given the reliance on animal models in TBI research, minimizing scientific animal experimentation is essential.[5] This study aims to establish optimized antibody concentrations for neurotrauma research, providing a reference for future studies. By improving stain clarity and usability, these findings enhance IHC methodologies while promoting more efficient and ethical research practices.

Literature review

The pathophysiology of TBI

Comprehending the pathology of TBI is the first step in understanding how enhancement of the data analysis technique of IHC facilitates research on TBI pathology. A substantial amount of research has been conducted on TBI and its pathophysiology. Giordano & Lifshitz summarize this research, discussing the most common pathophysiology after diffuse TBI.[1-5]

Diffuse TBI occurs when a mechanically-induced force impacts multiple parts of the brain. This can occur when the brain receives an inadequate amount of oxygen (i.e., hypoxic brain injury) or when there is an excess accumulation of fluid in the brain (i.e., cerebral edema). Focal TBI, on the other hand, happens when a mechanically-induced force impacts a localized area of the brain. This can happen during a car accident when the frontal lobe is impacted as the head hits the windshield.

Giordano and Lifshitz[1] and Blennow *et al.*[2] state that the loss of consciousness and a transient extension and/or flexion of the forearms (i.e., the fencing response) are common signs immediately observable after diffuse TBI. However, they may not be observed in all cases of TBI. Common transient symptoms include immediate disorientation, slurred speech, and vomiting. However, it is necessary to note that these symptoms depend on the "parameters of the initiating mechanical force."[1(p14)] In other words, acute symptoms after TBI are defined by characteristics of the mechanism of the injury (i.e., how much force was applied to the brain, where was the impact, how much time did it take, for example).

According to Giza and Hovda, another common pathophysiology of TBI is mechanically induced neuronal membrane disruption which results in an efflux of potassium ions at the cellular level.[1,6] This increase in extracellular potassium levels leads to the depolarization of neurons and the release of glutamate, a prominent excitatory neurotransmitter. The release of glutamate exacerbates the efflux of potassium, which generates a positive feedback loop of excitation directly after impact. As a consequence, if the impact affected the brainstem, the neurons in the lateral vestibular nucleus would become activated, resulting in the fencing response.[1,6] Glucose production in the brain is also affected after impact. In total, Giordano and Lifshitz, supported by Kenzie et al. and Giza and Hovda, claim that the cellular processes in the brain after TBI consist of a "frenzy" of activity to control damage while subsequent pathophysiological activities evolve with the TBI.[1 (p15),6-7]

Other common forms of acute pathophysiology after TBI include diffuse axonal injury (DAI), which disturbs the original neuronal circuitry in the brain; vascular disruption/dysfunction in the form of hematomas; an altered neural circuitry, which affects the way nerves align and connect; and inflammation in the brain.[1,6]

Neuroinflammation, one of the most studied aspects of TBI, is a pathophysiological response to injury in the CNS that removes injurious stimulus and initiates the healing process. It involves the activation of glial cells, specifically astrocytes and microglia, which regulate functional recovery after TBI. Astrocytes form a glial scar–a physical and chemical barrier that forms in the CNS after an injury–around lesions of overtly damaged neurons, although these are more common in focal TBI.[1,8] More importantly, astrocytes promote several neurotrophic factors–chemicals involved in the revival of neurons and the reduction of glutamate, decreasing excitotoxicity.[1,8] Additionally, the activation of microglia, resident innate immune cells in the brain, is triggered immediately after impact. These microglia, according to McGinn *et al.* and Giordano & Lifshitz, undergo morphological and functional changes, and produce substances like chemokines or cytokines to either promote or inhibit inflammation.[1,8-9] When healthy (ramified) microglia, which are very branched and have a small soma (cell body), are activated, they may take two forms: rod microglia, often found in the cortex and hippocampus, are less branched and have an elongated soma; and amoeboid microglia, which are found throughout the brain, are also less branched and have a spherical soma. The exact functions of the different morphologies of microglia require additional research. Delayed development of neurodegenerative pathology may be due to the presence of activated microglia in the brains of patients with TBI years after injury.[1] Hence, it is important to note, while inflammation may be acutely protective, in the long-term, inflammation may exacerbate the injury.[1]

More research is necessary to understand the individual mechanisms that constitute the pathophysiology of TBI.[1] Pathophysiology and the resultant symptoms can be present acutely and chronically; moreover, these pathophysiological processes often occur simultaneously and interdependently, highlighting the complexity of TBI's pathophysiology.[1, 7] Figure 2 further demonstrates the complexity of the pathophysiology of TBI.

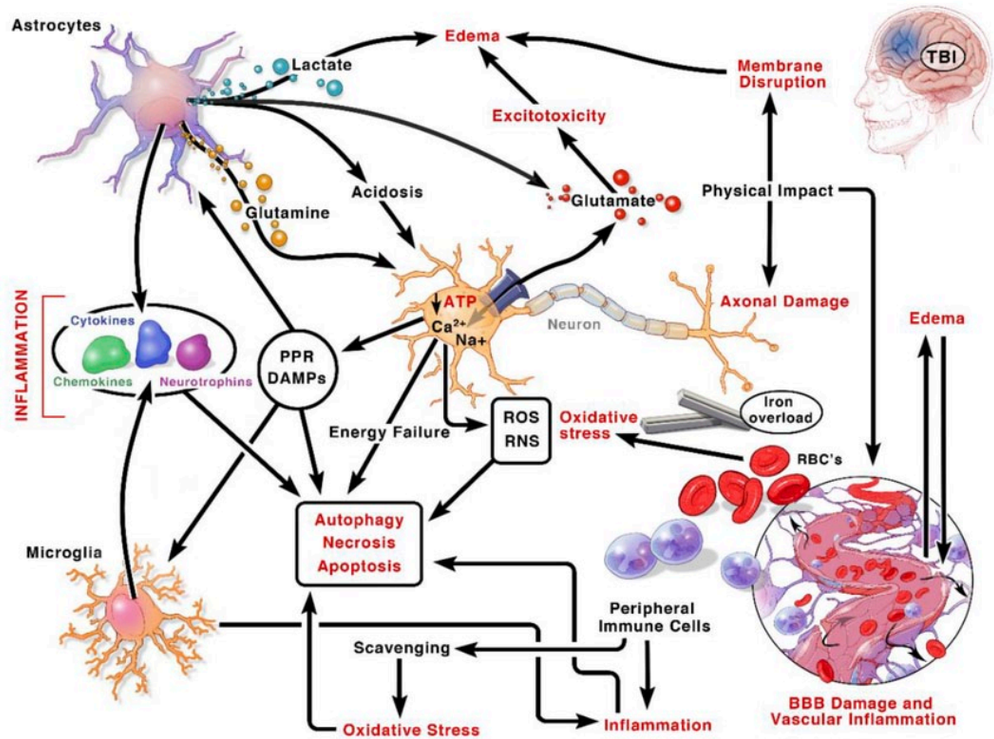

Figure 2. *Map of cellular processes that occur in the brain post-TBI.*[10]

Immunohistochemistry in neurotrauma & neuroinflammation

As mentioned, neuroinflammation has a prominent role in the acute and chronic pathophysiology of TBI. To study neuroinflammation, specifically cells such as microglia and astrocytes, researchers use IHC. Figure 3 illustrates two types of IHC techniques: direct immunoassay, which involves only one primary antibody that binds onto the antigen, and indirect immunoassay, which involves a secondary antibody binding onto the primary antibody. Direct and indirect immunoassays differ in their use of visualization methods: direct immunoassays typically utilize a substrate-enzyme binding process, such as horseradish peroxidase (HRP) with a substrate that emits visible light, while indirect immunoassays may employ fluorescence, where a fluorescent tag is attached to the secondary antibody to visualize the binding.

The diagram compares direct and indirect immunoassay as types of IHC. Direct immunoassay involves the direct binding of a primary antibody, attached to a visualization agent, horseradish peroxidase (HRP) enzyme. Indirect immunoassay involves the additional binding of a secondary antibody, attached to the HRP enzyme, to the primary antibody.

Because this study considers both primary and

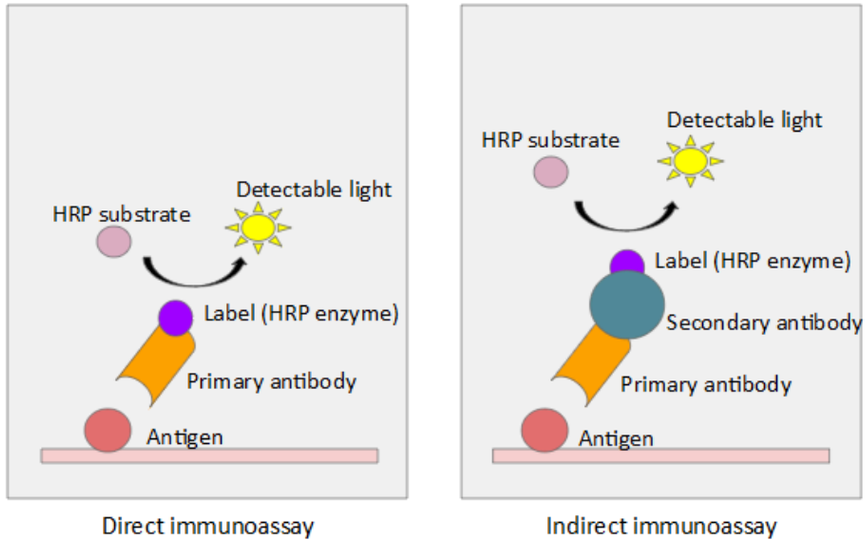

Figure 3. *Comparison of direct and indirect immunoassays in immunohistochemistry (IHC) using the example of horseradish peroxidase (HRP).*[11]

secondary antibodies, it is useful to understand the advantages and challenges of direct and indirect immunoassay. While indirect immunoassay is more complicated as it is difficult to find combinations of primary and secondary antibodies and their respective concentrations that optimize visualization, it can be used for all antigens. It may amplify the signal further, making it easier to visualize stains.[12] Signal amplification occurs through the possibility of a primary antibody being bound by multiple secondary antibodies, intensifying visible light emission.[12] On the contrary, direct immunoassay is easier to investigate, but it can only be used for highly expressed antigens as weakly expressed antigens are not visible without the significant amplification provided only by indirect immunoassay.[12]

IHC protocols involve several steps to ensure tissue sample clarity and reliability, including blocking, fixation, and antigen retrieval. Blocking prevents nonspecific antibody binding (i.e., the binding of the antibody onto molecules other than the targeted antigen), clarifying the image of the tissue sample.[13] Fixation is the process which preserves tissue by stabilizing cellular structures through protein binding.[13] Antigen retrieval then restores the antigenicity–i.e., the ability of an antigen to bind to an antibody–by severing the cross-links among antigens and fixatives.[13] While these steps can be manipulated to affect the visualization of the tissue, this study focuses on the impact of antibody concentrations on tissue visualization because they are one of the most essential yet under documented aspects of IHC.

Scientists have used IHC in the past to study the pathophysiology after TBI. For instance, Neri *et al.* conducted an IHC evaluation of Aquaporin-4 (AQP4)–an antigen found on astrocytes–and its correlation with Cluster of Differentiation 68 (CD68), Ionized calcium-binding adapter molecule 1 (IBA-1), Hypoxia-inducible factor 1-α (HIF-1α), Glial fibrillary acidic protein (GFAP), and Cluster of Differentiation 15 (CD15)–antigens found on glial cells and hypoxic cells–to study neuroinflammation after fatal TBI.[14] This technique determined the stages in time when microglia and astrocytes changed from a ramified to an activated state, whether astrocytes were associated with a beneficial role post-injury, and how these stains correlated with other pathophysiologic processes.[14] Similarly, Tanaka *et al.* investigated neuroinflammation after TBI in progranulin-deficient mice. Progranulin is a growth factor that regulates immune responses; by studying progranulin-deficient mice, researchers determine the role of progranulin in TBI's pathology by understanding what occurs with its absence. Tanaka *et al.* found correlations among different chemicals and cells, such as activated microglia, to conclude that progranulin suppresses excessive inflammatory responses related to active microglia in mice post-TBI.[15] Furthermore, Smith *et al.*, who used IHC to find temporal and spatial changes in the pattern of IBA1 and CD68 staining in rat brains post-TBI, visualized the pathophysiological effects of methamphetamine after severe TBI.[16] Finally, Stankov *et al.*, using a double staining IHC method for CD68 and IBA-1, understood more comprehensively the prevalence of cells like microglia and their morphology after brain contusions.[17] Hence, while fundamentally, Neri *et al.*, Tanaka *et al.*, Smith *et al.*, and Stankov *et al.* conducted different studies, they demonstrate the usefulness of IHC as a technique to understand the pathophysiology of TBI and brain injuries in general.[14-17]

The optimization of the visualization of IHC tissue samples

Optimizing tissue visualization in IHC involves several factors, including antibody concentrations, incubation time, temperature, and antibody selection.[12,18] This study specifically focuses on optimizing primary and secondary antibody concentrations for investigating post-TBI pathophysiology, as antibody concentration is a critical determinant of staining quality. In contrast, factors such as antigen retrieval and blocking serve as supplementary refinements. Additionally, while other aspects of IHC optimization are well-documented, research on antibody concentration optimization remains limited. By optimizing antibody concentrations, this study aims to improve IHC stain visualization, enhancing neuroinflammation and neurotrauma research.

Pre-existing guidelines for researchers conducting IHC are summarized by Magaki *et al.* They claim that the antibody concentration that will give the most clear, visible, and usable staining is determined by multiple dilutions of that concentrated antibody.[18] They recommend starting with the dilution recommended by the manufacturer and testing one dilution above and below it. For instance, if the manufacturer recommends a dilution of 1:400 (one part antibody to 400 parts solution), then testing 1:200, 1:400, and 1:800 dilutions will likely show an optimal visualization in one of the tested dilutions.[18] Because this study analyzed primary and secondary antibodies, it also considered the combinations of each primary antibody with different secondary antibodies, and their respective concentrations when optimizing the visualization of IHC tissue samples.

Gap

While general guidelines on IHC exist, there is a gap in the literature regarding specific antibody concentrations recommended for optimal visualization in neurotrauma. Researchers can find some antibody concentrations by investigating previous papers, but this is tedious and often futile. As a result, they resort to testing various antibody concentrations using the general guidelines outlined above on non-experimental tissue samples, subsequently using animal tissue and antibodies that could have been used for data collection. Researchers additionally tend to stop when their tissue samples meet their immediate needs, potentially overlooking valuable data. Optimizing IHC tissue samples would maximize data extraction from each sample, possibly leading to unexpected findings.

This paper aims to construct a reference for researchers unsure of what antibody concentrations they should use to optimize the visualization of their IHC tissue samples. Specifically, it focuses on antibodies involved in neurotrauma research, a field that heavily utilizes animal models, thereby underscoring the importance of developing methods that minimize redundant scientific animal experimentation.[5]

Method

Lab protocol

This study conducted IHC analyses on specific antibodies most prominent in neurotrauma research following Magaki *et al.*'s recommendation to test the manufacturer-recommended dilution–along with one dilution above and below it–to find the optimal primary and secondary antibody concentrations.[18]

Primary antibodies that bind to a specific antigen

are *anti*-[antigen], and secondary antibodies that bind to particular primary antibodies are *anti*-[primary antibody]. In this study, primary antibodies were raised in a species other than the species of the tissue to prevent nonspecific binding; nonspecific binding would occur as the secondary antibody would have a tendency to bind to the primary antibodies *and* the immunoglobulins–antibodies–naturally present in the tissue. Because rat tissue was used, primary antibodies were raised in a different species (e.g., *rabbit* anti-IBA1 antibodies). Primary antibodies that bind to the following antigens located on neuroinflammatory targets were used; the respective cell/cell regions on which they are mainly found are also provided:

- IBA1: microglia
- AQP4: astrocytes
- GFAP: glial cells
- Microtubule-Associated protein 2 (MAP2): neurons, oligodendrocytes (type of glial cell involved in the production of myelin in the CNS), astrocytes
- Myelin A1 antibody (MBP): myelin sheath (protective and insulating layer around nerve fibers), oligodendrocytes
- CD83: microglia

Primary Antibodies	Primary Antibody Manufacturer	**Citations of Antibody**	**Dilutions from Papers/ Manufacturer**	**Dilutions Tested in This Study**	**IHC Staining References**
Anti-IBA1	FUJIFILM Wako Pure Chemical Corporation[19]	Tanaka et al.[25]	1:100; 1:500 - 1:1000	1:100; 1:250; 1:500; 1:1000	Sroor et al.[32]
Anti-GFAP	Chemicon[20]	Lu et al.[26]	1:1,500; 1:400 - 1:800	1:500; 1:1000; 1:1500	Finney et al.[33]
Anti-MBP	Chemicon[21]	Clark et al., Song et al.[27,28]	1:2000; 1:500	1:200; 1:500; 1:1000	Hickey et al., Song et al., Clark et al.[27,28,34]
Anti-AQP4	Santa Cruz Biotechnology[22]	Han et al.[29]	1:100; 1:50-1:500	1:50; 1:100; 1:500	Tourdias et al., Han et al., Mesentier-Louro [35,29,36]
Anti-CD83	Thermo Fisher Scientific[23]	N/A	1:50-1:200	1:50; 1:100; 1:200	Arbab et al.[37]
Anti-MAP2	BioLegend[24]	Wang et al., Shelton et al.[30,31]	1:500 & 1:1000; 1:200 - 1:1000	1:250, 1:500, 1:1000	Guo et al.[38]

Table 1. *Summary of primary antibodies used in this study: Manufacturers, citations, dilution recommendations, tested dilutions, and IHC staining references for pathology assessment*
Abbreviations*: Anti-IBA1, Anti-Ionized Calcium-Binding Adapter Molecule 1; Anti-GFAP, Anti-Glial Fibrillary Acidic Protein; Anti-MBP, Anti-Myelin Basic Protein; Anti-AQP4, Anti-Aquaporin 4; Anti-CD83, Anti-CD83 Cell Surface Glycoprotein; Anti-MAP2, Anti-Microtubule-Associated Protein 2.*
Note: *Four concentrations are listed for IBA1 because concentrations 1:100, 1:250, and 1:500 were tested for the control batch and 1:100, 1:250, and 1:1000 were tested for the experimental batch.*

This project used secondary antibodies raised in a host species different from the primary antibodies (e.g., horse anti-rabbit for rabbit anti-[antigen]) to ensure proper binding, as antibodies recognize foreign antigens. Two secondary antibodies—horse anti-mouse and horse anti-rabbit—were used, with selection based on species compatibility with the primary antibody. The choice of secondary antibody did not affect the study's primary objective of evaluating antibody concentration effects on IHC tissue sample visualization but rather ensured specific binding to the corresponding primary antibody.

The tissue was fixed/embedded in paraformaldehyde and frozen in optimal cutting temperature (OCT)–a thick liquid at room temperature–because of pre-existing lab conventions. Natural horse serum (NHS) was used as the blocking serum because a blocking serum must originate from the same species in which the secondary antibody was raised, and both secondary antibodies were raised in a horse; proteins in this serum bind to natural immunoglobulins in the tissue, preventing primary and secondary antibodies from binding to non-target proteins. This project utilized a substrate-enzyme binding process–chromogenic staining–with 3,3 -diaminobenzidine (DAB) as the chromogen/substrate; DAB was chosen over fluorescence for its greater stability and longevity. A summary of the protocol, excluding the phosphate-buffered saline (PBS) washes which stabilize the pH and maintain tissue sample integrity after each step, is provided below:

IHC protocol (summary)

1. Removal of slide from the freezer and rehydration with PBS.
2. Antigen retrieval with sodium citrate buffer to unmask epitopes–the part of an antigen to which an antibody attaches itself.
3. Blocking to prevent non-specific binding.
4. Addition of primary antibodies to target the antigen of interest.
5. Addition of secondary antibody to bind to the primary antibody.
6. Blocking of endogenous peroxidases to avoid false-positive results.
7. Addition of Avidin-Biotin Complex (ABC) solution for signal amplification.
8. Application of DAB solution for visualization of antibody binding.
9. Dehydration of the sample and cover slipping using CitraSolv.

The rats in this study underwent diffuse traumatic brain injury (TBI) using the midline fluid percussion injury (mFPI) model, with an impact intensity of 320–340 mV—sufficient to induce TBI pathology without being fatal. Brain tissue was collected at seven and 28 days post-injury (DPI), ensuring that most samples within each antigen cohort originated from the same brain. This approach minimizes variability in pathology prevalence across compared tissue samples.

Secondary Antibodies	**Secondary Antibody Manufacturer**	**Citations of Antibody**	**Dilutions from Papers/ Manufacturer**	**Dilutions Tested in This Study**
Horse anti-mouse	Vector Laboratories[39]	Wen et al.[41]	1:200; 1:150 - 1:750	1:250; 1:500; 1:1000
Horse anti-rabbit	Vector Laboratories[40]	Moreno-Martinez et al.[42]	1:200; 1:150 - 1:750	1:250; 1:500; 1:1000

Table 2. *Summary of secondary antibodies used in this study: Manufacturers, citations, dilution recommendations, and tested dilutions*
Note: *IHC staining references are not included because secondary antibodies are used to enhance staining intensity, whereas primary antibodies bind to the antigen to indicate specific pathology. For the control IBA1, secondary antibody concentrations of 1:100, 1:250, 1:500 were used. After the results, the study shifted the concentration range to 1:250, 1:500, 1:1000 for the experimental antigens. See the "Results" section for further explanation.*

Analysis

Because the definition of a "visually optimized" tissue sample varies between individuals, this study employed an objective scoring system to assess immunohistochemically stained samples. The primary and secondary antibody concentrations determine antigen binding and, consequently, stain visibility. Insufficient concentrations may result in weak staining, while excessive concentrations can obscure tissue details. The goal is to identify the optimal antibody concentrations that yield clear, usable stains for analysis.

Scoring Guide for Optimized Tissue Samples

Score each of the following factors on a scale from 1 to 10:

1. Discernible pathology (DP): Contrast between cells and background, ensuring clear visualization of cellular processes.
2. Background noise (BN): Presence of non-specific staining that may obscure target cells.
3. Uniform staining (US): Consistency of staining across the tissue sample.

Calculate the final score using the following formula:

Final Score = (0.55 × DP) + (0.25 × (10 − BN)) + (0.20 × US)

The highest-scoring sample is considered visually optimized, and its corresponding antibody concentration(s) are identified as optimal for the given tissue and cell type.

A weighted average was taken as opposed to a normal average to prioritize pathology visualization (DP), which directly impacts neurotrauma research, over background noise (BN) and uniform staining (US). Background noise was weighted higher than uniform staining since excessive noise can obscure cellular processes, whereas non-uniform staining primarily affects large-area analyses, which are less common in TBI studies.

Following standard TBI research methodologies regarding DP and BN, this study focused on the primary somatosensory barrel cortex (S1BF) of the rat brain, a region with substantial TBI pathology. This targeted approach minimizes extraneous variables and enhances reproducibility. Nonetheless, including uniform staining in the evaluation allows for applicability to broader TBI pathology research involving larger tissue areas.

IBA1	P100 S100	P100 S250	P100 S500	P250 S100	P250 S250	P250 S500	P500 S100	P500 S250	P500 S500
Background noise	7	6	6	3	2	1.5	3.5	1.5	2
Discernible pathology	4	6	7	7.5	7.5	7.5	6	8	3
Uniform staining	7	6	6	7	7	7	5.5	6.5	7
Final score	4.35	5.5	6.05	7.275	7.525	7.65	6.025	7.825	5.05

Table 3. *Antibody concentration scores for IBA1 (control) Staining*
Abbreviations: *IBA1, Ionized Calcium-Binding Adapter Molecule 1; P, primary antibody; S, secondary antibody.*
Note: *The highest final score and its corresponding antibody concentrations are bolded. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.*

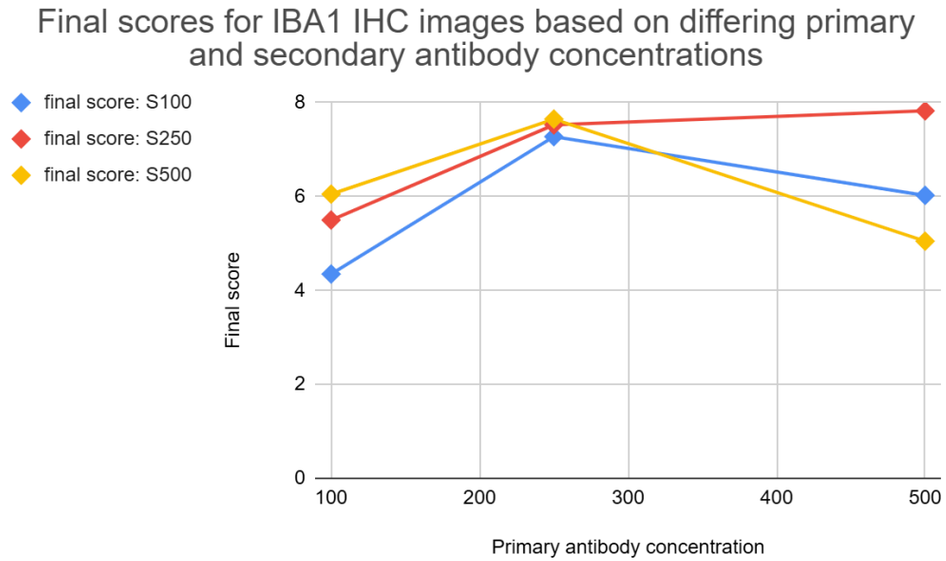

Figure 4. *Final scores for IBA1 (control) IHC images based on differing primary and secondary antibody concentrations.*

Results

IBA1: Control

The laboratory conducting this research specializes in microglia studies and has previously optimized antibody concentrations for the antigen IBA1. To validate the current methodology, this study re-optimized these concentrations. Consistent with prior findings, the optimal primary and secondary antibody concentrations were confirmed as 1:500 and 1:250, respectively. Rabbit anti-IBA1 served as the primary antibody, and horse anti-rabbit served as the secondary antibody. Control IBA1 IHC was performed using 7 DPI rat brain tissue.

Figures 5, 9, 13, 17, 21, 25, and 29 present images selected from different hemispheres to avoid dark spots, folding, tears, and other tissue integrity issues that could affect staining uniformity. These sections, chosen for strong tissue preservation, highlight varying levels of discernible pathology and background noise in IHC samples across different antibody concentrations. Figures 6, 10, 14, 18, 22, 26, and 30 specifically illustrate differences in staining uniformity.

IBA1: Experimental

Previous research performed IHC targeting the antigen IBA1 using secondary antibody concentrations of 1:100, 1:250, and 1:500 (see Figures 4–6) to validate the methodology. To further confirm these findings and maintain consistency with protocols used for other antigens, this study performed IHC targeting IBA1 with secondary antibody concentrations of 1:250, 1:500, and 1:1000, and primary concentrations of 1:250, 1:500, and 1:1000. Primary concentrations were selected around the previously optimized concentration of 1:500. Consistent with earlier results, the optimal primary and secondary concentrations were confirmed as 1:500 and 1:250, respectively. Experimental IHC for IBA1 was conducted using 28 DPI rat brain tissue.

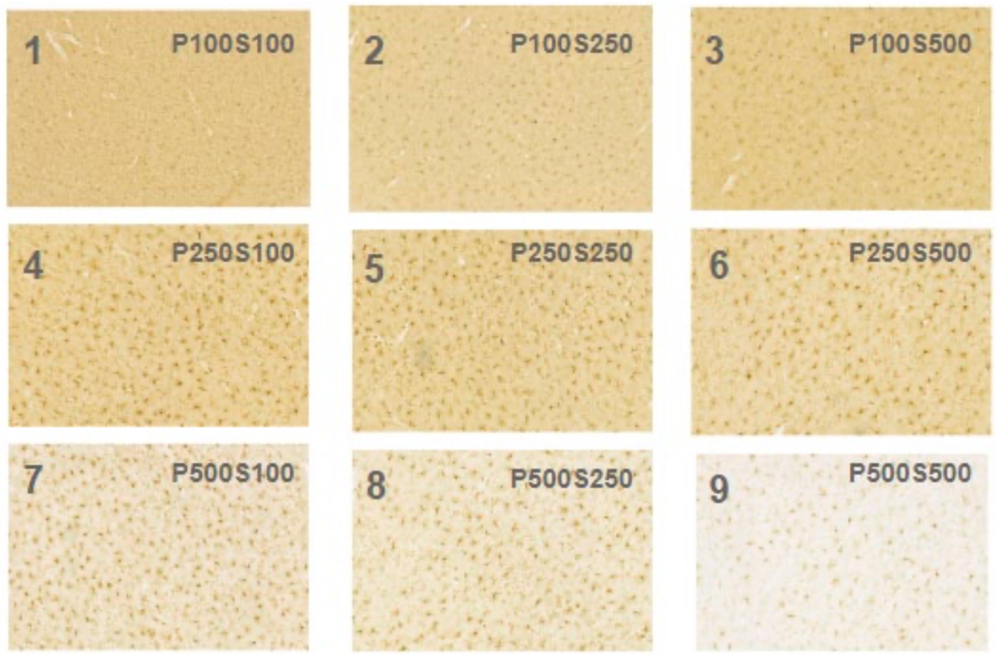

Figure 5. *IHC images targeting the antigen IBA1 (control).* **1**, P100S100, left hemisphere. 2, P100S250, right hemisphere. 3, P100S500, right hemisphere. 4, P250S100, left hemisphere. 5, P250S250, left hemisphere. 6, P250S500, right hemisphere. 7, P500S100, left hemisphere. 8, P500S250, right hemisphere. 9, P500S500, right hemisphere. All images are of the S1BF region magnified 13-14x. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

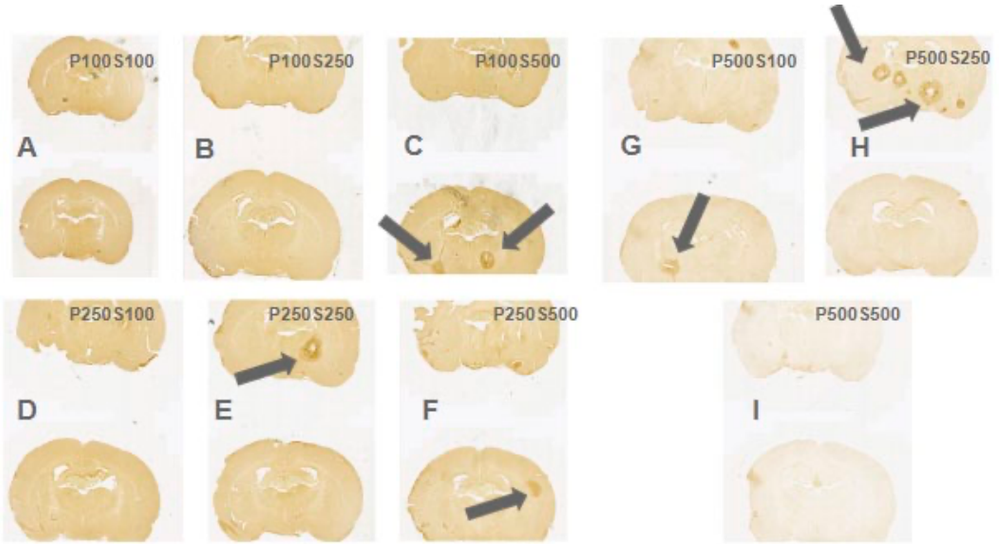

Figure 6. *Macroscopic pictures of IBA1 (control) tissue samples that demonstrate varying levels of uniform staining.* **A**, P100S100, US score = 7. **B**, P100S250, US score = 6. **C**, P100S500, US score = 6. **D**, P250S100, US score = 7. **E**, P250S250, US score = 7. **F**, P250S500, US score = 7. **G**, P500S100, US score = 5.5. **H**, P500S250, US score = 6.5. **I**, P500S500, US score = 7. Arrows indicate dark spots, which worsen the uniform staining score of the respective image. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

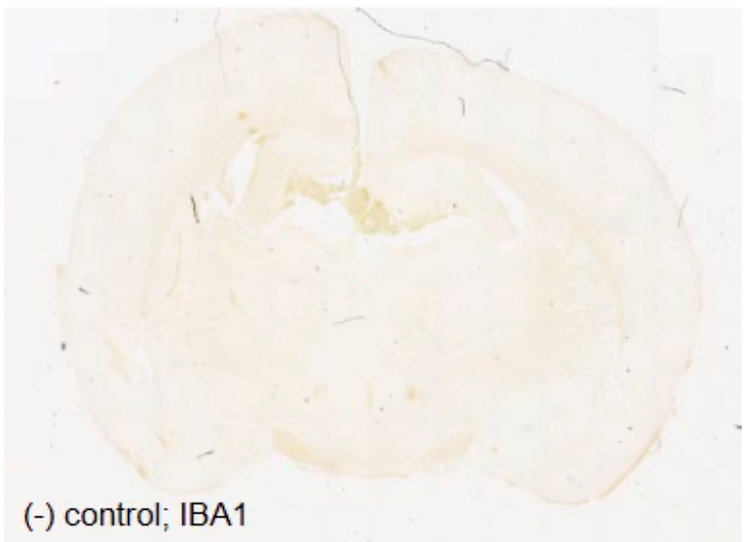

Figure 7. ***IBA1 – Negative control for comparison to the IHC images. The primary antibody is not applied, but the lowest concentration of the secondary antibody (Horse anti-rabbit; 1:500) is.***

IBA1	P250 S250	P250 S500	P250 S1000	**P500 S250**	P500 S500	P500 S1000	P1000 S250	P1000 S500	P1000 S1000
Background noise	5	7.5	8	4	6	7.5	3	3.5	3
Discernible pathology	6	5.5	5.5	6.5	6	5.5	5.5	6	6
Uniform staining	7	6	5	6.5	8	7	7	7	6
Final score	5.95	4.85	4.525	**6.375**	5.9	5.05	6.175	6.325	6.25

Table 4. *Antibody concentration scores for IBA1 (experimental) Staining*
Abbreviations: *IBA1, Ionized Calcium-Binding Adapter Molecule 1; P, primary antibody; S, secondary antibody.*
Note: *The highest final score and its corresponding antibody concentrations are bolded. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.*

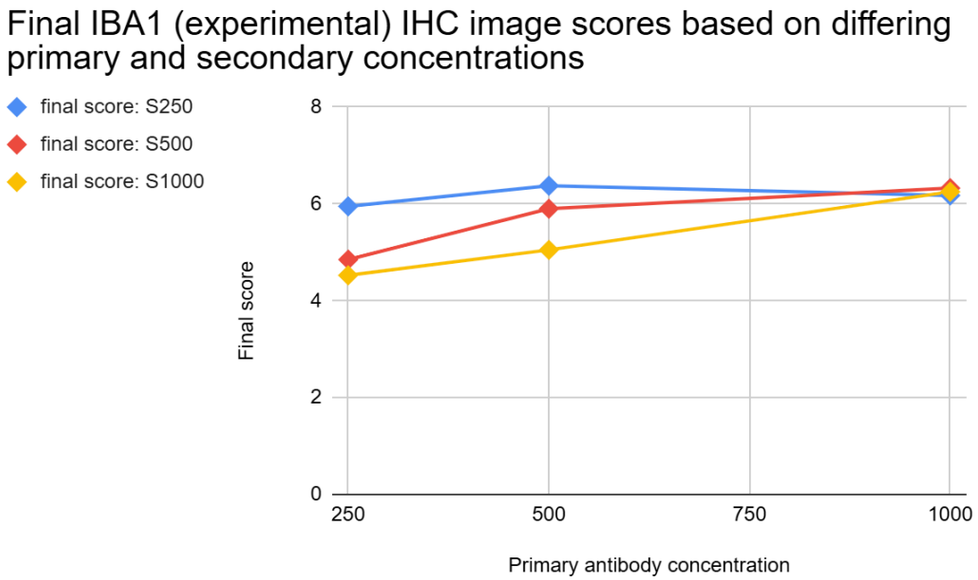

Figure 8. *Final scores for IBA1 (experimental) IHC images based on differing primary and secondary antibody concentrations.*

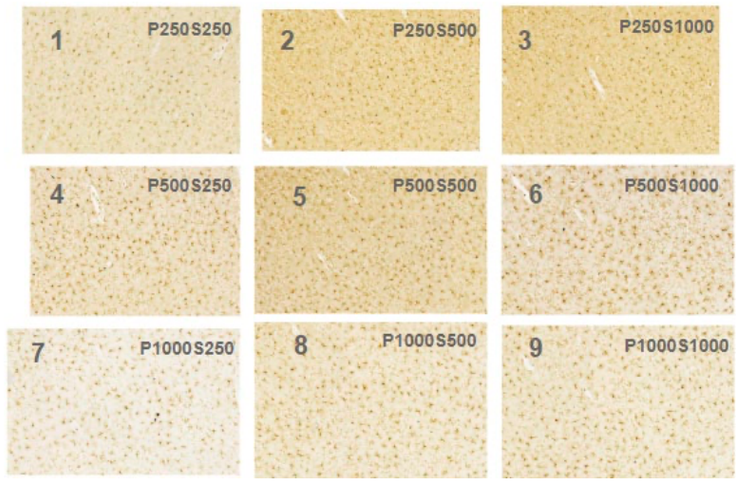

Figure 9. *IHC images targeting the antigen IBA1 (experimental).* **1**, P250S250, right hemisphere. **2**, P250S500, left hemisphere. **3**, P250S1000, left hemisphere. **4**, P500S250, left hemisphere. **5**, P500S500, left hemisphere. **6**, P500S1000, left hemisphere. **7**, P1000S250, left hemisphere. **8**, P1000S500, left hemisphere. **9**, P1000S1000, left hemisphere. All images are of the S1BF region and magnified 13-14x. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

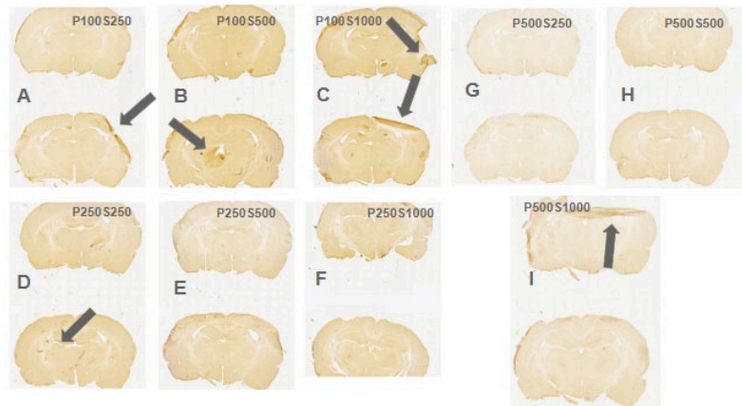

Figure 10. *Macroscopic pictures of IBA1 (experimental) tissue samples that demonstrate varying levels of uniform staining.* **A**, P250S250, US score = 7. **B**, P250S500, US score = 6. **C**, P250S1000, US score = 5. **D**, P500S250, US score = 6.5. **E**, P500S500, US score = 8. **F**, P500S1000, US score = 7. **G**, P1000S250, US score = 7. **H**, P1000S500, US score = 7. **I**, P1000S1000, US score = 6. Arrows indicate folding (**A, C, I**), fragmentation (**D, C**), and dark spots (**B**) which worsen the uniform staining score of the respective image. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

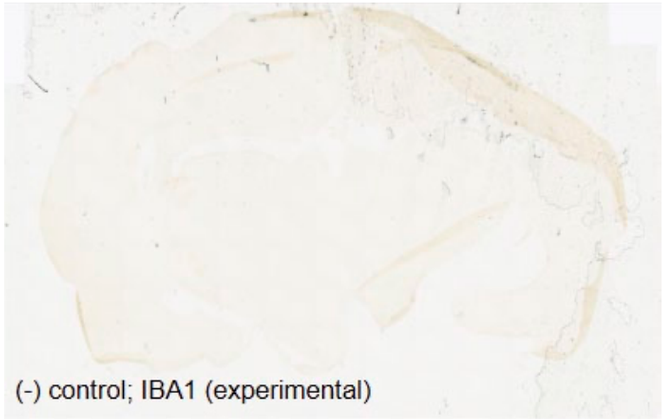

Figure 11. *IBA1 (experimental) – Negative control for comparison to the IHC images*. The primary antibody is not applied, but the lowest concentration of the secondary antibody (horse anti-rabbit; 1:1000) is.

GFAP

This study identified primary and secondary antibody concentrations of 1:1000 and 1:500, respectively, as optimal for visualizing the antigen GFAP. Mouse anti-GFAP served as the primary antibody, and horse anti-mouse served as the secondary antibody. GFAP IHC was performed using 7 DPI rat brain tissue.

GFAP	P500 S250	P500 S500	P500 S1000	P1000 S250	**P1000 S500**	P1000 S1000	P1500 S250	P1500 S500	P500 S1000
Background Noise	4	5	4	5	4.5	5.5	3.5	4	3
Discernible pathology	5	6	5	4	7	6	4	6.5	2
Uniform staining	4	4	7	7	4	4	4	4	7
Final score	5.05	5.35	5.15	4.85	**6.025**	5.225	4.625	5.875	4.25

Table 5. *Antibody concentration scores for GFAP staining*
Abbreviations: *GFAP, Glial Fibrillary Acidic Protein; P, primary antibody; S, secondary antibody.*
Note: *The highest final score and its corresponding antibody concentrations are bolded. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P1000S500 indicates a primary antibody concentration of 1:1000 and a secondary antibody concentration of 1:500.*

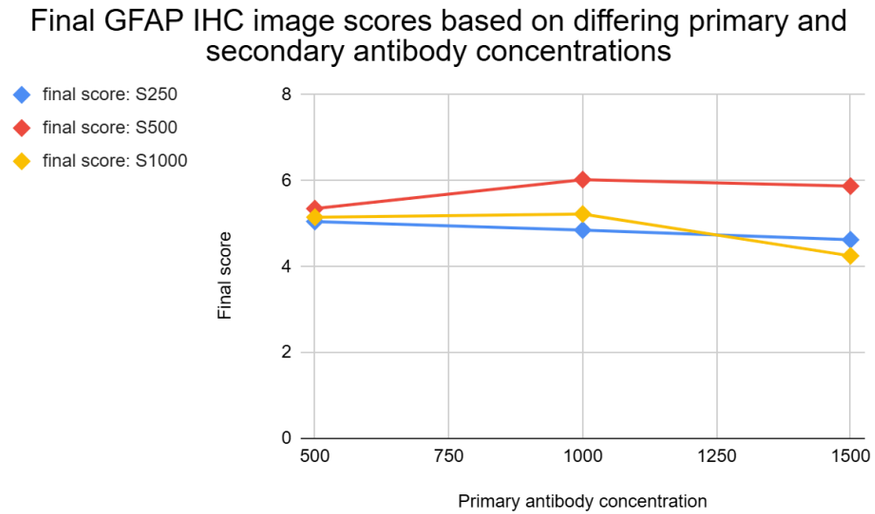

Figure 12. *Final scores for GFAP IHC images based on differing primary and secondary antibody concentrations.*

Figure 13. *IHC images targeting the antigen GFAP.* **1**, P500S250, right hemisphere. **2**, P500S500, right hemisphere. **3**, P500S1000, right hemisphere. **4**, P1000S250, left hemisphere. **5**, P1000S500, right hemisphere. **6**, P1000S1000, right hemisphere. **7**, P1500S250, left hemisphere. **8**, P1500S500, left hemisphere. **9**, P1500S1000, left hemisphere. All images are of the S1BF region and magnified 13-14x. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

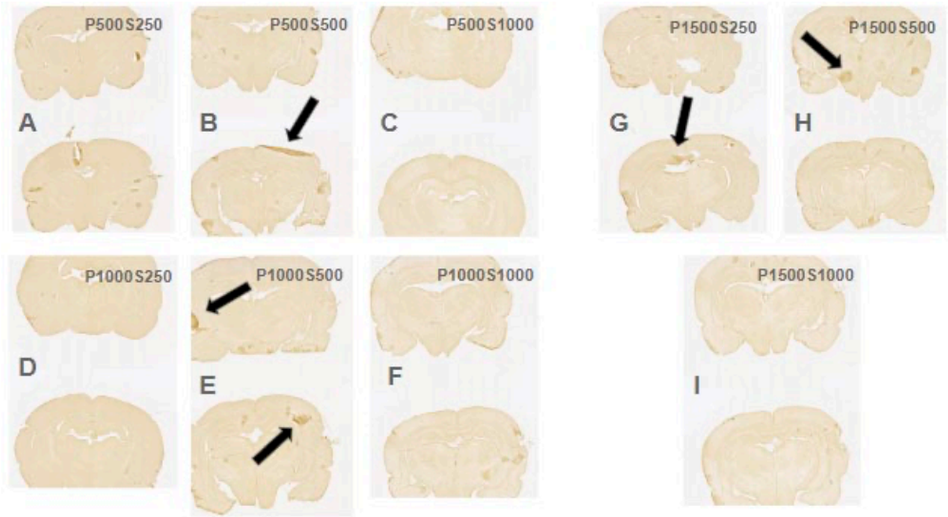

Figure 14. *Macroscopic pictures of GFAP tissue samples that demonstrate varying levels of uniform staining.* **A**, P500S250, US score = 4. **B**, P500S500, US score = 4. **C**, P500S1000, US score = 7. **D**, P1000S250, US score = 7. **E**, P1000S500, US score = 4. **F**, P1000S1000, US score = 4. **G**, P1500S250, US score = 4. **H**, P1500S500, US score = 4. **I**, P1500S1000, US score = 7. Arrows indicate dark spots (**E, G, H**) and folding (**B**), which worsen the uniform staining score of the respective image. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

Figure 15. *GFAP – Negative control for comparison to the IHC images.* The primary antibody is not applied, but the lowest concentration of the secondary antibody (horse anti-rabbit; 1:1000) is.

MAP2	P250 S250	P250 S500	P250 S1000	P500 S250	P500 S500	P500 S1000	P1000 S250	**P1000 S500**	P1000 S1000
Background Noise	3	4	2.5	5	4.5	5	3.5	4	2.5
Discernible pathology	3.5	6	3	6	6.5	5.5	6.5	7	3
Uniform staining	6.5	6.5	7	5	7	6.5	6.5	6	7
Final score	4.975	6.1	4.925	5.55	6.35	5.575	6.5	**6.55**	4.925

Table 6. *Antibody concentration scores for MAP2 staining*
Abbreviations: *MAP2, Microtubule-Associated Protein 2; P, primary antibody; S, secondary antibody.*
Note: *The highest final score and its corresponding antibody concentrations are bolded. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P1000S500 indicates a primary antibody concentration of 1:1000 and a secondary antibody concentration of 1:500.*

MAP2

A primary antibody concentration of 1:1000 and a secondary antibody concentration of 1:500 produced optimal staining for the MAP2 antigen. Mouse anti-MAP2 served as the primary antibody, and horse anti-mouse served as the secondary antibody. IHC was performed for MAP2 using 7 DPI rat brain tissue.

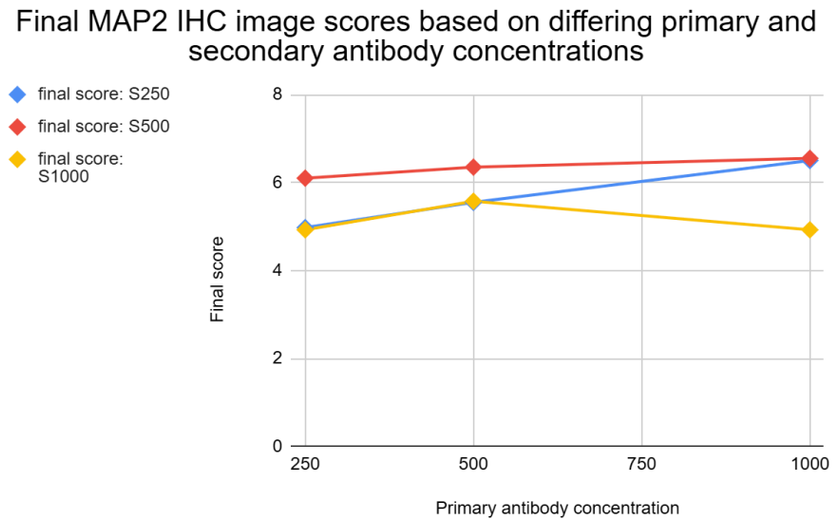

Figure 16. *Final scores for MAP2 IHC images based on differing primary and secondary antibody concentrations.*

Figure 17. *IHC images targeting the antigen MAP2.* **1**, P250S250, right hemisphere. **2**, P250S500, left hemisphere. **3**, P250S1000, left hemisphere. **4**, P500S250, left hemisphere. **5**, P500S500, right hemisphere. **6**, P500S1000, right hemisphere. **7**, P1000S250, right hemisphere. **8**, P1000S500, left hemisphere. **9**, P1000S1000, left hemisphere. All images are of the S1BF region and magnified 13-14x. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

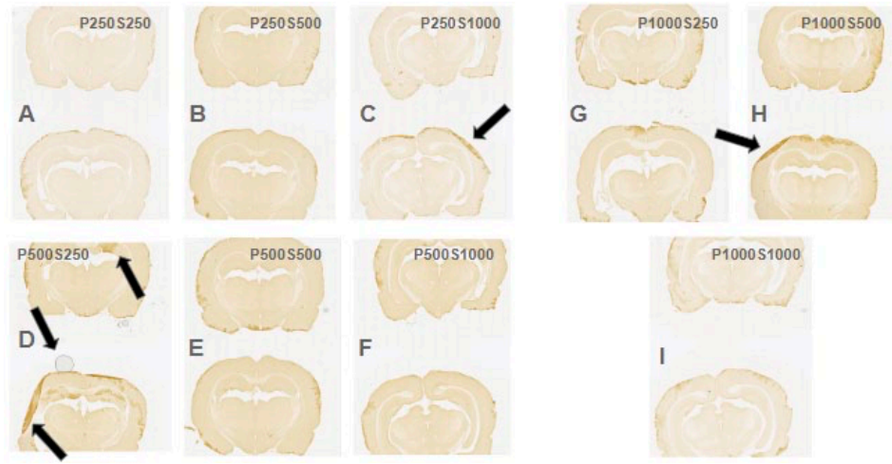

Figure 18. *Macroscopic pictures of MAP2 tissue samples that demonstrate varying levels of uniform staining.* **A**, P250S250, US score = 6.5. **B**, P250S500, US score = 6.5. **C**, P250S1000, US score = 7. **D**, P500S250, US score = 5. **E**, P500S500, US score = 7. **F**, P500S1000, US score = 6.5. **G**, P1000S250, US score = 6.5. **H**, P1000S500, US score = 6. **I**, P1000S1000, US score = 7. Arrows indicate dark spots (**D**), folding (**D, C, H**), and air bubbles (**D**), which worsen the uniform staining score of the respective image. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

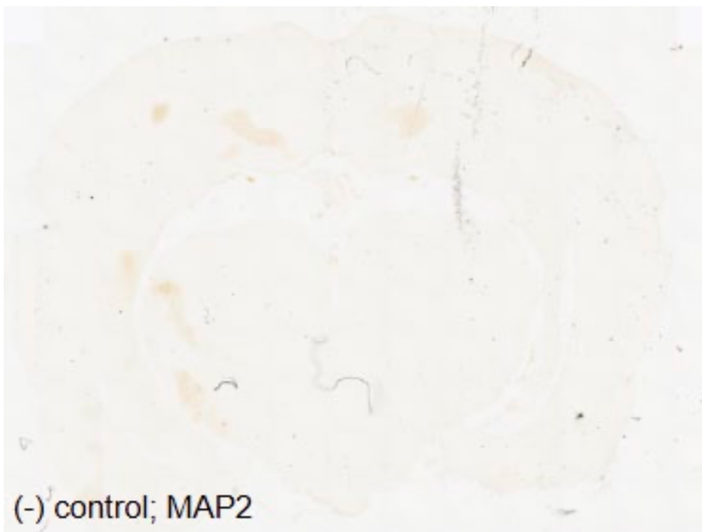

Figure 19. *MAP2 – Negative control for comparison to the IHC images*. The primary antibody is not applied, but the lowest concentration of the secondary antibody (horse anti-mouse; 1:1000) is.

AQP4

This study found that a primary antibody concentration of 1:50 and a secondary antibody concentration of 1:500 produced optimal staining for the AQP4 antigen. Mouse anti-AQP4 served as the primary antibody, and horse anti-mouse served as the secondary antibody. AQP4 IHC was performed on 7 DPI rat brain tissue using secondary concentrations of 1:250 and 1:500, and on 28 DPI tissue using a secondary concentration of 1:1000.

AQP4	P50 S250	**P50 S500**	P50 S1000	P100 S250	P100 S500	P100 S1000	P500 S250	P500 S500	P500 S1000
Background Noise	5	5	3	3.5	1.5	2	1.5	1.5	1
Discernible pathology	5	5.5	3	3.5	1.5	2	1.5	1	1
Uniform staining	4.5	7	7	7.5	8	6.5	8	8	8
Final score	4.9	**5.675**	4.8	4.628125	4.55	4.4	4.55	4.275	4.4

Table 7. *Optimized antibody concentrations for AQP4 staining*
Abbreviations: *AQP4, Aquaporin-4; P, primary antibody; S, secondary antibody.*
Note: *The highest final score and its corresponding antibody concentrations are bolded. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P50S500 indicates a primary antibody concentration of 1:50 and a secondary antibody concentration of 1:500.*

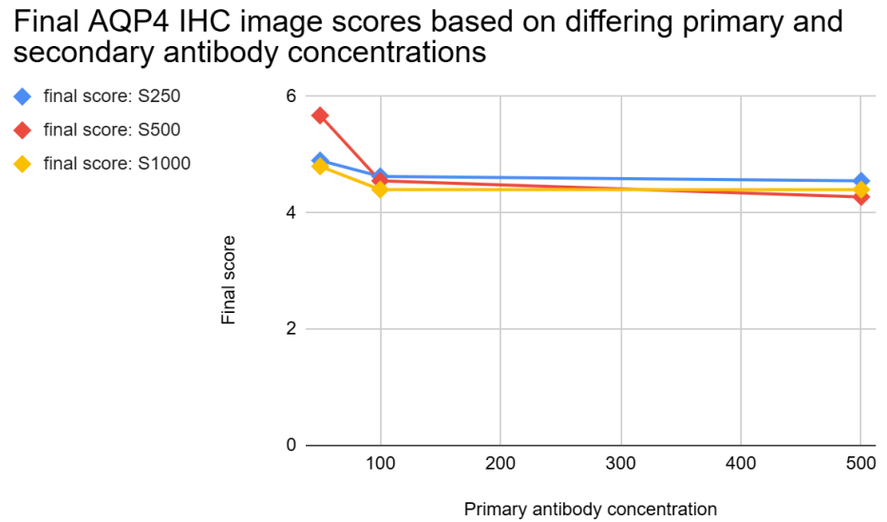

Figure 20. *Final scores for AQP4 IHC images based on differing primary and secondary antibody concentrations.*

Figure 21. *IHC images targeting the antigen AQP4.* **1**, P50S250, right hemisphere. **2**, P50S500, left hemisphere. **3**, P50S1000, left hemisphere. **4**, P100S250, left hemisphere. **5**, P100S500, left hemisphere. **6**, P100S1000, right hemisphere. **7**, P500S250, right hemisphere. **8**, P500S500, right hemisphere. **9**, P500S1000, left hemisphere. All images are of the S1BF region and magnified 13-14x. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

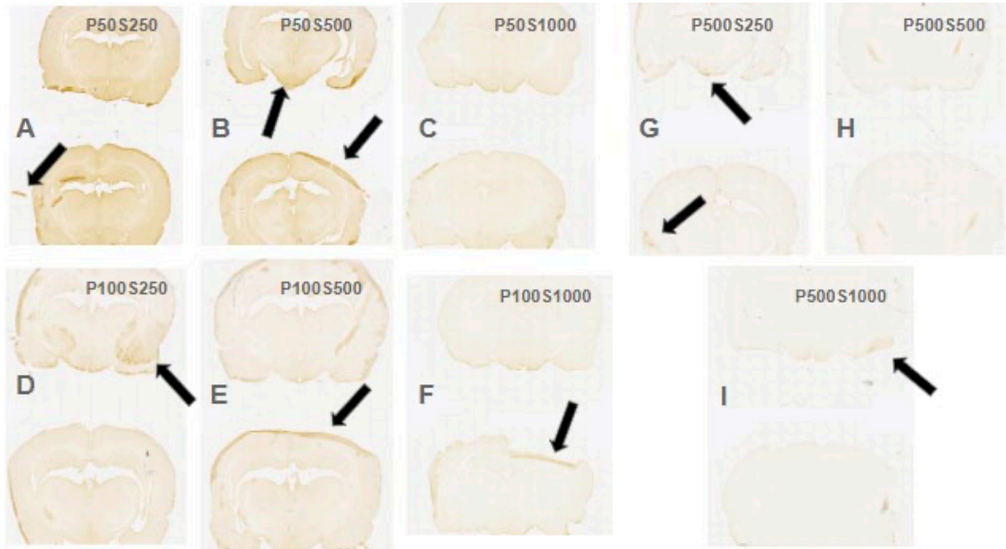

Figure 22. *Macroscopic pictures of AQP4 tissue samples that demonstrate varying levels of uniform staining.* **A**, P50S250, US score = 4.5. **B**, P50S500, US score = 7. **C**, P50S1000, US score = 7. **D**, P100S250, US score = 7.5. **E**, P100S500, US score = 8. **F**, P100S1000, US score = 6.5. **G**, P500S250, US score = 8. **H**, P500S500, US score = 8. **I**, P500S1000, US score = 8. Arrows indicate dark spots (**B, D, I, G**), folding (**E, F**), and fragmentation (**A**), which worsen the uniform staining score of the respective image. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

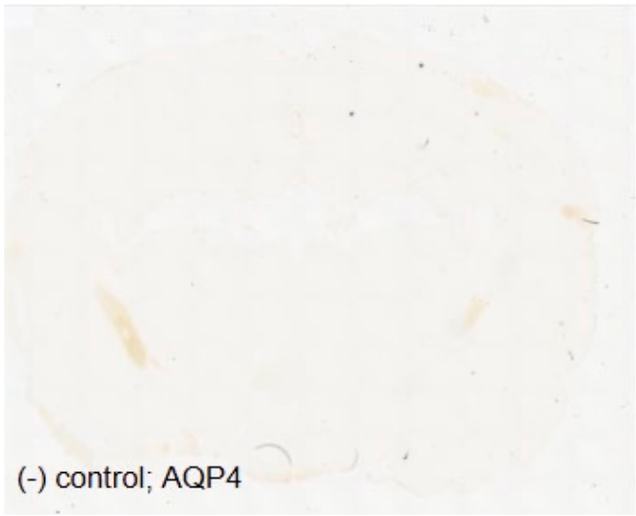

Figure 23. *AQP4 – Negative control for comparison to the IHC images.* The primary antibody is not applied, but the lowest concentration of the secondary antibody (horse anti-mouse; 1:1000) is.

CD83	P50 S250	**P50 S500**	P50 S1000	P100 S250	P100 S500	P100 S1000	P200 S250	P200 S500	P200 S1000
Background Noise	7	5	2.5	7	2.5	5.5	5	5	2
Discernible pathology	6	7	2.5	6	3	6.5	6.75	6	4
Uniform staining	7	6	6	8	8	7	3	3	1
Final score	5.45	**6.3**	4.45	4.45	5.125	6.1	5.5625	5.15	4.4

Table 8. *Antibody concentration scores for CD83 staining*
Abbreviations: *CD83, Cluster of Differentiation 83; P, primary antibody; S, secondary antibody.*
Note: *The highest final score and its corresponding antibody concentrations are bolded. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P50S500 indicates a primary antibody concentration of 1:50 and a secondary antibody concentration of 1:500.*

CD83

A primary antibody concentration of 1:50 and a secondary antibody concentration of 1:500 produced optimal staining for the CD83 antigen. Rabbit anti-CD83 served as the primary antibody, and horse anti-rabbit served as the secondary antibody. 7 DPI tissue samples were used to target the CD83 antigen.

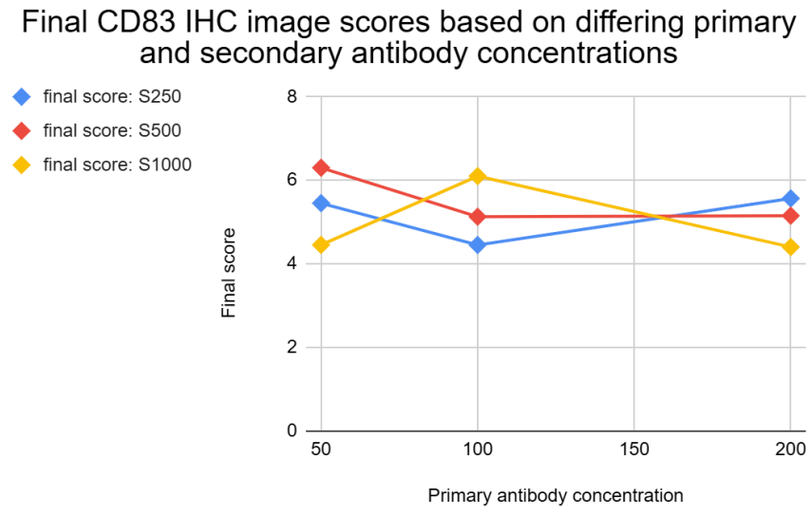

Figure 24. *Final scores for CD83 IHC images based on differing primary and secondary antibody concentrations.*

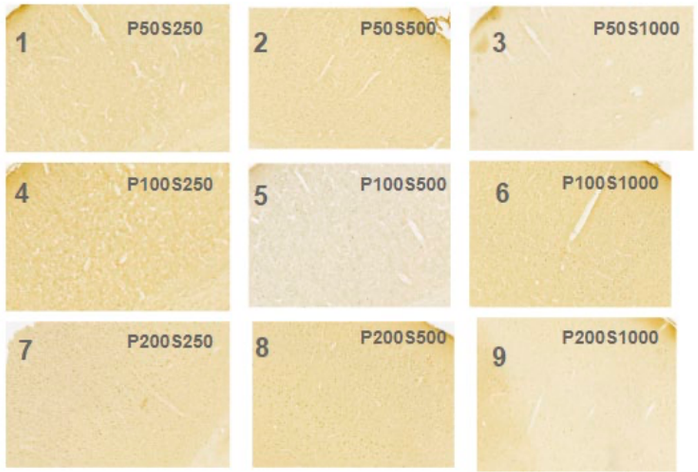

Figure 25. *IHC images targeting the antigen CD83.* **1**, P50S250, left hemisphere. **2**, P50S500, right hemisphere. **3**, P50S1000, left hemisphere. **4**, P100S250, left hemisphere. **5**, P100S500, left hemisphere. **6**, P100S1000, right hemisphere. **7**, P200S250, left hemisphere. **8**, P200S500, right hemisphere. **9**, P200S1000, right hemisphere. All images are of the S1BF region and magnified 13-14x. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

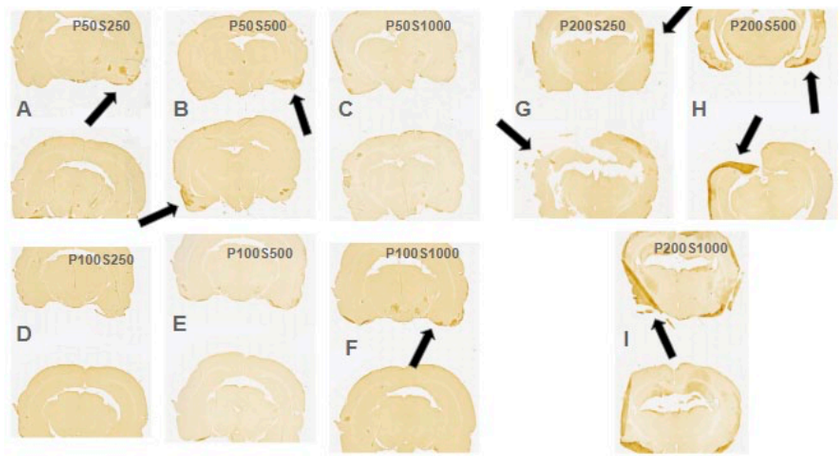

Figure 26. *Macroscopic pictures of CD83 tissue samples that demonstrate varying levels of uniform staining.* **A**, P50S250, US score = 7. **B**, P50S500, US score = 6. **C**, P50S1000, US score = 6. **D**, P100S250, US score = 8. **E**, P100S500, US score = 8. **F**, P100S1000, US score = 7. **G**, P200S250, US score = 3. **H**, P200S500, US score = 3. **I**, P200S1000, US score = 1. Arrows indicate dark spots (**A, B, F, G, H**), folding (**H, I**), and fragmentation (**G, I**), which worsen the uniform staining score of the respective image. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

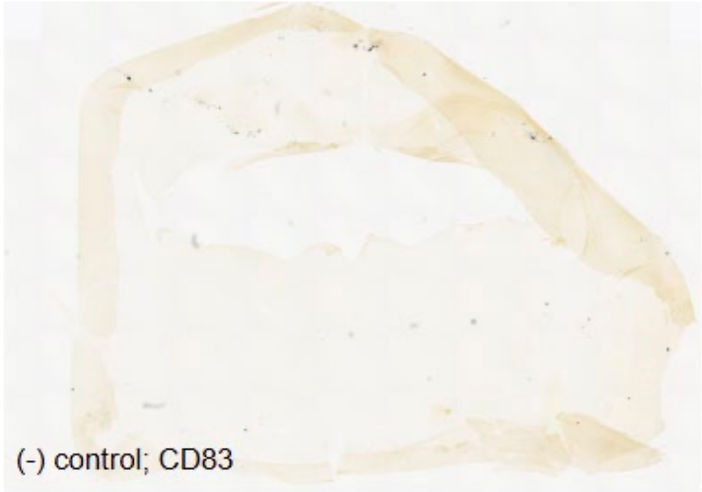

Figure 27. *CD83 – Negative control for comparison to the IHC images.* The primary antibody is not applied, but the lowest concentration of the secondary antibody (horse anti-rabbit; 1:1000) is.

MBP

This study found that a primary antibody concentration of 1:1000 and a secondary antibody concentration of 1:250 produced optimal staining for the MBP antigen. Rabbit anti-MBP served as the primary antibody, and horse anti-rabbit served as the secondary antibody. 28 DPI tissue samples were used to target the MBP antigen.

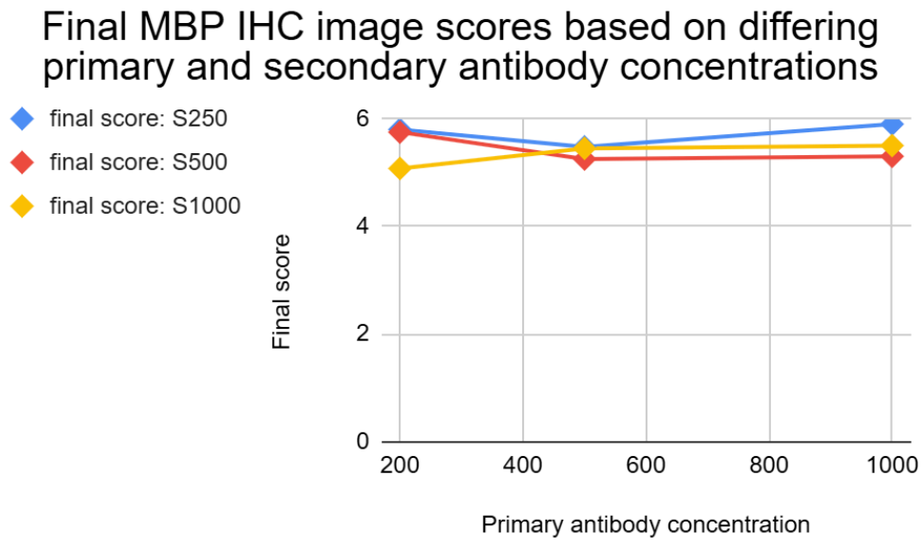

Figure 28. *Final scores for MBP IHC images based on differing primary and secondary antibody concentrations.*

MBP	P200 S250	P200 S500	P200 S1000	P500 S250	P500 S500	P500 S1000	**P1000 S250**	P1000 S500	P1000 S1000
Background Noise	4.5	5	5	6	4	4	5	3	3
Discernible pathology	5.5	6	5.5	6.5	5	5	7	5	5
Uniform staining	7	6	4	4.5	5	6	4	4	5
Final score	5.8	5.75	5.075	5.475	5.25	5.45	**5.9**	5.3	5.5

Table 9. *Antibody concentration scores for MBP staining*
Abbreviations: *MBP, Myelin Basic Protein; P, primary antibody; S, secondary antibody.*
Note: *The highest final score and its corresponding antibody concentrations are bolded. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P1000S250 indicates a primary antibody concentration of 1:1000 and a secondary antibody concentration of 1:250.*

Figure 29. *IHC images targeting the antigen MBP.* **1**, P200S250, left hemisphere. **2**, P200S500, right hemisphere. **3**, P200S1000, left hemisphere. **4**, P500S250, left hemisphere. **5**, P500S500, right hemisphere. **6**, P500S1000, right hemisphere. **7**, P1000S250, right hemisphere. **8**, P1000S500, left hemisphere. **9**, P1000S1000, right hemisphere. All images are of the S1BF region and magnified 13-14x. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

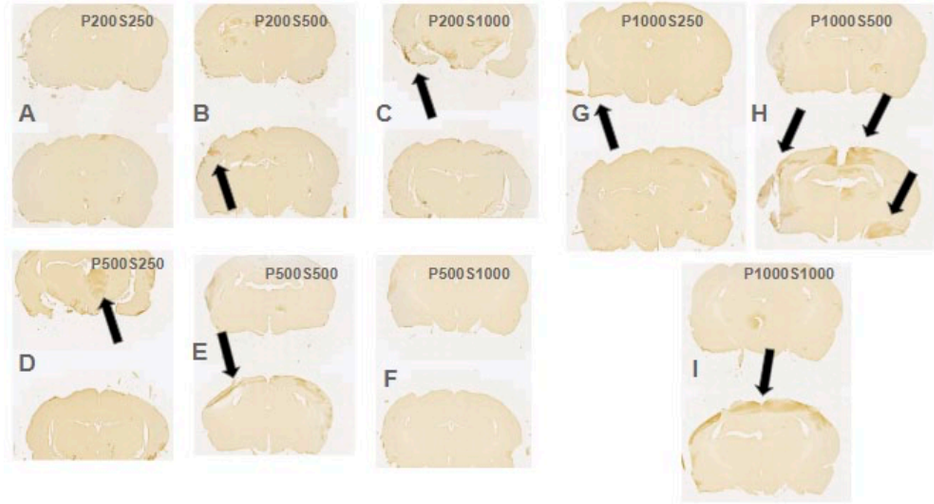

Figure 30. *Macroscopic pictures of MBP tissue samples that demonstrate varying levels of uniform staining.* **A**, 200S250, US score = 7. **B**, P200S500, US score = 6. **C**, P200S1000, US score = 4. **D**, P500S250, US score = 4.5. **E**, P500S500, US score = 5. **F**, P500S1000, US score = 6. **G**, P1000S250, US score = 4. **H**, P1000S500, US score = 4. **I**, P1000S1000, US score = 5. Arrows indicate dark spots (**D, B, C, H, I**), folding (**E, I**), and fragmentation (**G, H**), which worsen the uniform staining score of the respective image. Antibody concentrations are denoted as the ratio of one part antibody to parts of solution; e.g., P500S250 indicates a primary antibody concentration of 1:500 and a secondary antibody concentration of 1:250.

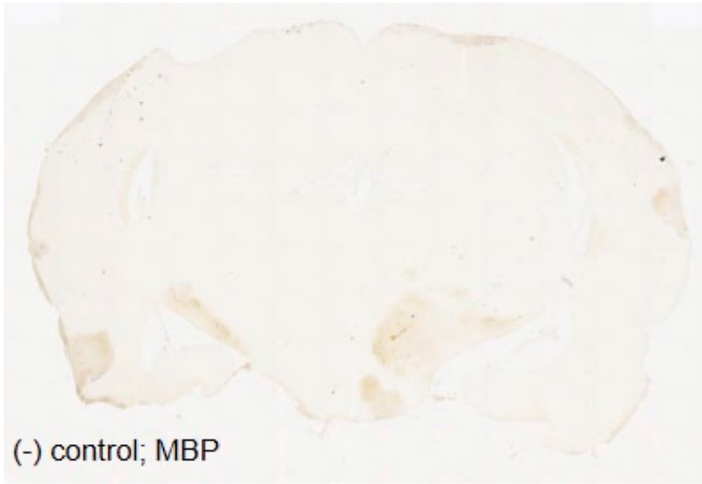

Figure 31. *MBP – Negative control for comparison to the IHC images*. The primary antibody is not applied, but the lowest concentration of the secondary antibody (horse anti-rabbit; 1:1000) is.

Discussion

Findings

Most stains followed a consistent trend: decreasing secondary antibody concentrations led to reduced staining intensity, which often correlated with more uniform staining, minimal background noise, and reduced discernible pathology. This outcome is expected, as lower secondary antibody concentrations reduce binding to the primary antibody, thereby diminishing signal intensity.

Several stains appeared visually similar—specifically, GFAP P100S500 (1:100 primary, 1:500 secondary) compared to GFAP P1500S500, and MAP2 P1000S250 compared to MAP2 P1000S500. To differentiate between these, previous studies were consulted to identify the specific pathological features that should be visible.[33,38]

This study prioritized the visibility of pathology. While other researchers may prefer MAP2 P1000S250 for applications requiring broader anatomical coverage due to its uniformity and low background, MAP2 P1000S500 received higher scores here due to its clearer pathological detail, which is particularly relevant for traumatic brain injury (TBI) research and its alignment with IHC images from prior literature.[38] Researchers should define their imaging goals—whether structural clarity or pathological detail—before selecting antibody concentrations.

While most stains followed the expected pattern, an unexpected result was observed with CD83 at a primary concentration of 1:100. As the secondary antibody concentration decreased, staining intensity initially decreased but then unexpectedly increased from P100S500 to P100S1000 (Figure 7). Similarly, the experimental IBA1 IHC batch deviated from the expected pattern. Although this could be due to extraneous variables, it offers new insight into secondary antibody concentrations and staining intensity. Future studies should replicate these experiments to determine whether these anomalies result from antibody concentration manipulation or other factors.

Optimal primary and secondary antibody concentrations showed no clear pattern across antigens. Various combinations of primary and secondary antibody concentrations yielded optimal results for each antigen, likely due the binding efficacy of different antibody types to distinct antigens. Except for CD83 and AQP4, the optimal primary antibody concentration for each antigen was either the lowest or moderate concentration tested. This aligns with the observation that increasing primary antibody concentration often increases background staining.

The deviation in CD83 may be due to the range of concentrations tested. Since no previous paper used the exact CD83 antibody product used in this research, the manufacturer-recommended concentration was used, which may have led to suboptimal visual results. Future research should test a broader

Antigen	Tissue samples	Primary antibody and optimized concentration	Secondary antibody and optimized concentration
IBA1 (C)	7 DPI	Rabbit anti-IBA1; 1:500	Horse anti-rabbit; 1:250
IBA1 (EXP)	28 DPI	Rabbit anti-IBA1; 1:500	Horse anti-rabbit; 1:250
GFAP	7 DPI	Mouse anti-GFAP; 1:1000	Horse anti-mouse; 1:500
MAP2	7 DPI	Mouse anti-MAP2; 1:1000	Horse anti-mouse; 1:500
AQP4	7 DPI and 28 DPI	Mouse anti-AQP4; 1:50	Horse anti-mouse; 1:500
CD83	7 DPI	Rabbit anti-CD83; 1:50	Horse anti-rabbit; 1:500
MBP	28 DPI	Rabbit anti-MBP; 1:1000	Horse anti-rabbit; 1:250

Table 10. *Optimized primary and secondary antibody concentrations for various antigens*

Abbreviations: C, control. EXP, experimental.

concentration range to confirm this. AQP4 findings suggest that more concentrated antibody concentrations may provide visual optimization, challenging current literature on AQP4 antibody concentrations and providing new insights.

Secondary antibody concentrations of 1:1000 were not optimal, likely due to insufficient staining intensity. Initial IBA1 testing showed that secondary antibody concentrations above 1:100 resulted in excessive background noise, leading to a revised range of 1:250–1:1000 for experimental antigens. As the data show, optimal secondary antibody concentrations vary by antigen, though they were consistently 1:250 or 1:500 in this study. Future studies should explore a broader range of secondary antibodies, as current literature primarily uses 1:200, contrary to this study's findings, and variability across antigens should be considered. A wider range of primary antibody concentrations should also be tested to determine if an optimal concentration exists outside those explored in this study.

Scoring in this study was antigen-specific; scores should not be compared across different antigens. For example, while the optimized MBP image received a score of 5.9 and MAP2 received 6.55, this does not indicate that MAP2 optimization was superior.

Limitations

This study aimed to objectively determine optimal antibody concentrations but has several limitations.

Scoring Subjectivity: While quantitative scoring was used, some subjectivity remains. Cell counting methods could improve objectivity, but resource limitations prevented their inclusion in this study. Future research should incorporate these techniques.

Variability in IHC Protocols: Tissue folding and fragmentation occasionally occurred, likely due to processing variability or chance errors. Extraneous factors, such as hydrophobic barrier (PAP) pen efficacy, temperature fluctuations, and pH imbalances, may have influenced staining outcomes. Replicating protocols is recommended to determine whether optimized results are due to concentration changes or external variables.

Limited Antigen Selection: Due to resource constraints, this study focused on a subset of antigens relevant to neurotrauma research. Expanding antigen selection in future studies could benefit a wider research audience, potentially extending beyond neurotrauma research.

IHC Staining Technique: This study used DAB staining for its stability and ease of storage. However, fluorescence IHC staining is also widely used in neurotrauma research for its ability to provide high resolution images and detect multiple proteins simultaneously. Future studies should determine optimal antibody concentrations for fluorescence IHC.

Lack of Secondary Antibody Literature: Current literature primarily recommends a standard 1:200 concentration for secondary antibodies, regardless of primary concentration or technique. This study provides new insight, showing that optimal secondary concentrations vary by antigen, emphasizing the need for further research across antigens and staining methods.

Primary Antibody Range: While primary antibody concentrations have more extensive literature, variability in optimal concentrations suggests that broader testing is warranted, especially for antibodies like CD83 and AQP4, where the optimal concentration may be outside the tested range.

Tissue Consistency: Resource constraints prevented using the same DPI tissue across all antigens. AQP4 IHC required tissue obtained at different post-injury time points. Both 7 DPI and 28 DPI were selected because pathology is present at both stages with minimal differences, which likely had negligible impact on research outcomes.[43] However, slight variations may exist, and future studies should standardize tissue selection across antigens to minimize extraneous variables and better understand antigen-specific staining patterns and cellular interactions post-injury. It is important to note that this study focused on optimizing staining for each antigen, rather than multiple injury time points. The primary limitation is the inability to use multiple timepoints of injury for *each* antigen, with a focus on pathology at specific time points instead. ***Implications & Future Directions***

This study has important implications for neurotrauma research. By identifying optimal antibody concentrations for key antigens, it enhances IHC data analysis while promoting the ethical principle of reducing animal use. Improved IHC methodologies maximize data extraction from fewer samples, minimizing unnecessary scientific animal experimentation.

Additionally, this study offers novel insight into immunohistochemistry, particularly regarding secondary antibody dilutions, CD83 and AQP4 concentrations, and the relationship between secondary concentrations and staining intensity. Researchers are encouraged to further investigate these areas. Future directions include:

- Incorporating quantitative analysis methods to improve objectivity.
- Investigating mediating factors between antibody concentrations and image quality.
- Optimizing additional aspects of IHC, such as antigen retrieval and blocking procedures.
- Modifying the scoring guide to prioritize different image features and appeal to a broader research audience.
- Expanding applications to TBI pathology studies, including cross-analysis with immunocytochemistry, MRI, and Western blot.

Despite the substantial prevalence of TBI in sports and the military, its pathology remains unexplored. Incremental advancements in research are critical for deepening the understanding of TBI, developing therapeutic treatments, and improving patient outcomes. This study aims to contribute to that broader effort, emphasizing both the scientific and societal significance of advancing TBI research.

Ethics Statement

All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Arizona College of Medicine and followed the guidelines of the National Institute of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize suffering and the quantity of animals used.

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Appendix

This appendix contains a general IHC protocol template and an example completed protocol for IBA1 to demonstrate the specifics of the IHC protocols used by this study.

Appendix A: IHC protocol template for each antigen

Date: | Basic [antigen] Protocol with Antigen Retrieval & Blocking

Project: optimization

Notes:

Primary antibody: [Name of primary antibody]

Secondary antibody: [Name of secondary antibody]

Day 1 ( )

Remove slides from -80° freezer and put in -20° freezer → 15 min

Remove slides from -20° freezer at RT→ 15 min

Place slides on a slide warmer at 50° → 60 min

Rehydration:

1X PBS (3 x 5 min)

Antigen Retrieval:

Place slides in sodium citrate buffer (pH 6.0)

Microwave: Power 6 for 3 min, then Power 3 for 10 min

Note: Place two dishes into microwave, other dish can be filled with water

Let slides cool for 60 min (or cool to the touch)

1X PBS wash (3 x 5 min)

PAP Pen

Block:

10% NHS serum, 0.3% Triton-100 in 1X PBS, 300 ul/slide = incubate for 60 min

NHS → ([total volume of liquid])(0.10) = [microliters of NHS used]

Triton → ([total volume of liquid])(0.003) = [microliters of Triton used]

PBS → total ul - (NHS + Triton) = [microliters of PBS used]

1X PBS wash (3 x 5 min)

Primary antibody:

NOTE: negative control gets no primary; continue with blocking solution or PBS overnight

Optimization of primary in 1% NHS serum, 0.1% Triton-100 in 1X PBS, 300 ul/slide → RT overnight

	Primary antibody	NHS	Triton	PBS
S dilution				
M dilution				
L dilution				

DAY 2 ( )

1X PBS wash with 0.1% Tween (3 x 5 min)

Secondary Antibody:

Optimized secondary in 4% N__S serum, 0.4% Triton-100 in 1XPBS → 60 min (300 ul/slide)

NOTE: put the negative control secondary for in the largest dilution category

	Secondary antibody	NHS	Triton	PBS
S dilution				
M dilution				
L dilution				

1X PBS wash with 0.1% Tween (3 x 5 min)

Block endogenous peroxidases

Place slides in 9 ml of H202 in 200 ml 1X PBS → 20 minutes (300 ul/slide)

Watch for bubbles. If none appear, H202 is bad

Make ABC solution

Needs to sit in the fridge for 30 minutes before application

Place 2 drops A + 2 drops B in a falcon tube containing 5 ml 1X PBS

1X PBS wash with 0.1% Tween (3 x 5 min)

Apply ABC, 300 ul/slide → 30 min

1X PBS wash with 0.1% Tween (3 x 5 min)

DAB

Make DAB solution according to the instructions on the box → 300 ul/slide

Look at about 2-4 minutes for each slide, wait for them to get dark → time dependent

DON'T FORGET: all trash must go into a falcon tube filled with 10 ml of bleach. All pipette tips and Kimwipes, etc. need to be placed into this falcon tube waste. Spray DAB with bleach when done.

Wash in tap water (5-10 min)

The ions in the tap water stop the DAB from processing

Dehydrate slides in EtOH to dehydrate the tissue

70% EtOH → 10 dips X2

90% EtOH → 10 dips X2

100 EtOH → 10 dips X2

Citrasolv #1 & Citrasolv #2 → 10 min each

Permount coverslip and leave in the hood to dry overnight

Clean with Xylene and long Q-tips the next day

Appendix B: Example completed IBA1 IHC protocol

Date: | Basic IBA1 Protocol with Antigen Retrieval & Blocking

Project: optimization

Notes:

Primary antibody: Rabbit anti-IBA1

Secondary antibody: Horse anti-rabbit

Day 1 ( )

Remove slides from -80° freezer and put in -20° freezer → 15 min

Remove slides from -20° freezer at RT→ 15 min

Place slides on a slide warmer at 50° → 60 min

Rehydration:

1X PBS (3 x 5 min)

Antigen Retrieval:

Place slides in sodium citrate buffer (pH 6.0)

Microwave: Power 6 for 3 min, then Power 3 for 10 min

Note: Place two dishes into microwave, other dish can be filled with water

Let slides cool for 60 min (or cool to the touch)

1X PBS wash (3 x 5 min)

PAP Pen

Block:

10% NHS serum, 0.3% Triton-100 in 1X PBS, 300 ul/slide = incubate for 60 min

NHS → (3000)(0.10) = 300 ul

Triton → (3000)(0.003) = 9 ul

PBS → total ul - (NHS + Triton) = 3000 - 309 = 2691 ul

1X PBS wash (3 x 5 min)

Primary antibody:

NOTE: negative control gets no primary; continue with blocking solution or PBS overnight

Optimization of primary in 1% NHS serum, 0.1% Triton-100 in 1X PBS, 300 ul/slide → RT overnight

	Primary antibody	NHS	Triton	PBS
1:250	3.6 ul	9 ul	0.9 ul	886.5 ul
1:500	1.8 ul	9 ul	0.9 ul	888.3 ul
1:1000	0.9 ul	9 ul	0.9 ul	889.2 ul

DAY 2 ( )

1X PBS wash with 0.1% Tween (3 x 5 min)

Secondary Antibody:

Optimized secondary in 4% NHS serum, 0.4% Triton-100 in 1X PBS → 60 min (300 ul/slide)

NOTE: put the negative control secondary for in the largest dilution category

	Secondary antibody	NHS	Triton	PBS
1:250	3.6 ul	36 ul	3.6 ul	856.8 ul
1:500	1.8 ul	36 ul	3.6 ul	858.6 ul
1:1000	1.2 ul	48 ul	4.8 ul	1146 ul

1X PBS wash with 0.1% Tween (3 x 5 min)

Block endogenous peroxidases

Place slides in 9 ml of H202 in 200 ml 1X PBS → 20 minutes (300 ul/slide)

Watch for bubbles. If none appear, H202 is bad

Make ABC solution

Needs to sit in the fridge for 30 minutes before application

Place 2 drops A + 2 drops B in a falcon tube containing 5 ml 1X PBS

1X PBS wash with 0.1% Tween (3 x 5 min)

Apply ABC, 300 ul/slide → 30 min

1X PBS wash with 0.1% Tween (3 x 5 min)

DAB

Make DAB solution according to the instructions on the box → 300 ul/slide

Look at about 2-4 minutes for each slide, wait for them to get dark → time dependent

DON'T FORGET: all trash must go into a falcon tube filled with 10 ml of bleach. All pipette tips and Kimwipes, etc. need to be placed into this falcon tube waste. Spray DAB with bleach when done.

Wash in tap water (5-10 min)

The ions in the tap water stop the DAB from processing

Dehydrate slides in EtOH to dehydrate the tissue

70% EtOH → 10 dips X2

90% EtOH → 10 dips X2

100 EtOH → 10 dips X2

Citrasolv #1 & Citrasolv #2 → 10 min each

Permount coverslip and leave in the hood to dry overnight

Clean with Xylene and long Q-tips the next day