# Glyoxal Fixation Allows Constant Staining of Brain Vessels, Pericytes, and Blood-Brain Barrier Proteins

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**Short Report**

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# Abstract

Brain vascular staining has great importance in understanding cerebrovascular pathologies. Paraformaldehyde 4% is considered the gold standard fixation technique for immunohistochemistry and it revolutionized the examination of proteins in fixed tissues. However, this fixation technique produces inconsistent immunohistochemical staining results due to antigen masking. Here we test a new fixation protocol using 3% glyoxal and demonstrate that this method provides overall better staining of the brain vasculature, pericytes, and tight junction proteins than 4% paraformaldehyde. Using this new fixation technique will provide more detailed information about vascular protein expressions, their distributions, and colocalizations with other proteins at the molecular level in the brain vasculature.


# 1. Introduction

There is no doubt that exploring proteins in cells is fundamental to understanding tissue and organ function. Snapshots of these biomolecules provide information about their distribution, expression, function, and colocalization with other proteins. The standard technique is based on the biochemistry of antibody labeling or fluorescent protein techniques. Innovative fluorescence-based technologies have been recently developed with the help of super-resolution microscopy to visualize proteins at the nano-molecular scale(1–3). However, after the current gold standard paraformaldehyde (PFA) fixation, immunohistochemical (IHC) staining of these cellular proteins using antibody or fluorophore binding remains problematic(4) .

The general objective of fixation is to preserve essential chemical and physical characteristics of tissue or cells by disabling proteolytic enzymes, inhibiting the growth and activity of microbes, and altering characteristics of the cells or tissues at the molecular level to improve their mechanical strength as well as stability (5–7). For immunostaining, an ideal fixation protocol should not reduce accessibility or affinity of antigens for antibodies or alter tissue or cell structure (8).

In brain vascular staining, PFA ensures protein immobilization and ultrastructure preservation but interferes with epitope recognition and penetration of antibodies, subsequently resulting in poor or inconsistent staining of proteins in the brain vessels (9). It remains critical to overcome this limitation as fundamental knowledge such as cerebral vascular development, blood-brain barrier (BBB) and neurovascular interactions rely heavily on this technique. Thus an optimal fixation method is required for generating consistent results on routine vascular stainings such as structural exposition, protein distribution, expression and can provide critical information on vascular functions.

Prior publications have recommended glyoxal as an alternative to PFA and report that it can act faster than PFA, cross-links proteins more effectively, and improves the preservation of cellular morphology (10). However, glyoxal fixation has not been tested for its efficiency in brain vascular staining. In this manuscript, we investigate glyoxal fixation as an alternative to PFA to determine whether it is a

dependable fixative for staining structural brain vascular networks, associated pericytes, and BBB/tight junction proteins.

## 2. Materials And Methods

# 2.1. Experimental Animals

C57BL/6 mice (weighing 25–30 g and age of 8–12 weeks) of both sexes, obtained from Jackson Laboratories, Farmington, CT USA, were used for this study. All mice were fed with a standard laboratory diet and water, maintained under standard laboratory conditions (temperature: 25 ± 2°C, humidity: 60 ± 5%, 12 h dark/light cycle) with free access to a standard pellet diet and water *ad libitum*. Animal procedures were carried out under the oversight of the Animal Care and Use Committee of the University of Texas Health Science Center at Houston (protocol # AWC-19-0120 ), and in strict compliance with National Institutes of Health guidelines.

# 2. 2. Materials used

PFA 4% (P6148) was purchased from Sigma-Aldrich (MilliporeSigma), MA, USA. Phosphate-buffered saline (PBS)(CA008-050) was obtained from Gen DEPOT Texas, USA. Permount mounting medium (SP15-500), hydrochloric acid ( A481212), and sodium hydroxide (S320-500) were purchased from Fisher Scientific, USA. Glyoxal solution A (16525 A) and glyoxal solution B (16525 B) were bought from Electron Microscopy Sciences, Hatfield, PA, USA. Gills hematoxylin (24243-500) was procured from Polysciences, PA, USA, and eosin Y (H-3502) was procured from Vector Laboratories, California, USA.

# 2.3. Preparation of PFA

PFA 4% was prepared in PBS (pH 7.4) with stirring and heating to approximately 60°C in a ventilated hood, then cooled and filtered. The pH was adjusted to 6.9 using 1N HCl and stored at 4°C for one month.

# 2.4. Preparation of Glyoxal

4 mL of 3% glyoxal was prepared by mixing 3.6 mL of solution A (16525 A, Electron Microscopy Sciences) with 313 µL of solution B (16525 B, Electron Microscopy Sciences).

# 2.5. Fixation and sectioning

Mice were kept under general anesthesia (isoflurane: 3% induction and 1% maintenance with 100% O2 as a carrier) and perfused (intracardiac) first with 5 mL PBS and then with 5 mL of 4% PFA. After perfusion, brain samples were collected and stored in 4% PFA at 4°C overnight and then transferred into PBS at 4°C for long-term storage.

For glyoxal fixation, after PBS perfused the brain was collected, washed with PBS, and kept in 3% glyoxal overnight at room temperature with rotation. For long-term storage, brain samples in glyoxal were stored at 4°C.

Extracted brains were then sectioned to 35-µm thick free-floating coronal slices using a semiautomatic vibratome (LEICA VT 1000S). Collected PFA sections were stored at 4°C in PBS till staining was done. Glyoxal sections were either used for staining or stored in glyoxal for long-term use.

## 2.6. H & E staining

Matching sections were taken from both PFA and glyoxal fixed brain samples into glass slides and sections were completely covered with hematoxylin for three minutes followed by rinsing in two changes in distilled water, each for 15 seconds, dipped in 100% ethanol for 10 seconds, drained off the excess ethanol and then eosin Y solution was added to the slides and incubated for 30 seconds. Then, sections were dehydrated using ascending grades of ethanol from 70–100%, cleared in xylene, and mounted with permount mounting medium (SP15-100 Fisher Scientific, USA).

## 2.7. Immunostaining

Brain samples were taken from both glyoxal and PFA fixed sections and observed under the bright field microscope (ZEISS Discovery. 12 steREO) to make ensure tissue morphology is retained through both fixation techniques. For IHC analysis, slices were washed thrice in PBS + 0.1% TritonX (PBST) and blocked for 1 h with 1% BSA. Each slice was then incubated overnight at 4°C with gentle shaking in PBST with primary antibody. The following antibodies were used: **Laminin** (1:200 Anti-Laminin α-2 Antibody (4H8-2): sc-59854; Santa Cruz Biotechnology, USA), **Isolectin B4** (1: 200 DL-1207 Vector Laboratories, California, USA), **Claudin 5** (1:200 4C3C2; Invitrogen, Massachusetts, United States) and **VE-cadherin** (1:200, 36-1900; Invitrogen, Massachusetts, United States), **Aquaporin-4** (1:400, A2887; ABclonal, Woburn, MA, USA) **Occludin** (1: 250, 00241; BiCell Scientific Missouri, USA) and **Zonula occludens-1** (1: 250, 00236; BiCell Scientific Missouri, USA), **Neural/glial antigen 2** (NG2) (1: 200 ab5320; Abcam, USA), **platelet-derived growth factor receptor beta** (PDGFRB) (1: 200 31695; Cell Signaling Technologies, Danvers, USA).

The following day, the brain slice was washed thrice with PBST. Sections stained with conjugated antibodies were mounted on glass slides using DAPI Fluoromount-G (SouthernBiotech Birmingham, AL) and imaged on a fluorescent microscope (Leica Thunder imager). Non-conjugated antibodies were then probed with a biotin-conjugated secondary antibody from Vector Laboratories in PBST with 1% serum for 1 h. The following secondary antibodies were used: Goat Anti-Rabbit IgG Antibody (H + L), Biotinylated (BA-1000-1.5), Goat Anti-Mouse IgG Antibody (H + L), and Biotinylated (BA-9200-1.5). Slices were then washed thrice in PBS, followed by 1 hr incubation with Streptavidin, DyLight® 488/594 (SA-5488-1/ SA-5549-1), washing, mounting, and imaging.

# 3. Results And Discussion

Recently many studies (11-15) have replaced PFA with glyoxal (3%), in tissue fixation because of the specific advantages associated with glyoxal over PFA. Advantages include its less toxic nature (16), faster reaction rates, selective control over cross-linking so that it can retain immunoreactivity

characteristics and allows to skip the antigen retrieval treatments (17), (18). Here we are investigating glyoxal for optimal brain fixation, multi-protein vascular, and pericyte staining procedures.

## 3.1. Brain architecture was preserved both in PFA and Glyoxal fixation

It is important to maintain the macroscopic architecture and microenvironment of the brain samples by following the appropriate fixation technique. In the current study, we compared PFA (4%) with glyoxal (3%) fixation in maintaining brain structure *in situ*. Previous studies by Bussolati et al., 2017 and Richter et al., 2018 (12, 18) have reported that glyoxal has rapid penetration properties. However, an overnight incubation is necessary for fixing the deep brain structures. In line with a previous study conducted by Iwashita et al., 2020 (19), in the present study it was noticed that mouse brain fixed with glyoxal exhibited lower stiffness than PFA (Figure. 1).

To evaluate and compare the preservation of brain anatomy across both PFA and glyoxal fixation conditions we used H&E staining. Both PFA and glyoxal were able to stain efficiently by H & E staining. As evident in Figure. 1 in whole coronal brain sections, brain structures, and morphology were well-preserved with each fixative. However, close observation revealed that the glyoxal fixed brain section appear more sharper, with a better resolution than PFA fixed brain sections.

## 3.2 Glyoxal fixation is optimal for obtaining consistent vascular staining in the brain.

To visualize blood vessels, immunohistochemical techniques targeting endothelial markers such as cluster of differentiation (CD) 31 (20, 21), basement membrane markers such as laminin (22), and lectins that bind to the luminal part of the capillary endothelium through interactions with sugar residues (23) have been widely used. Prior reports (24-26) and from our lab experience, currently used immunohistochemical vascular staining methods with 4% PFA fixed sections were not providing consistent results. This is probably due to the antigen masking from the high crosslinking during fixation. To overcome this, different antigen retrieval methods were used: for example, high-temperature treatment in citrate buffer and enzymatic treatment with pepsin. However, this adds time and cost consuming steps to the procedure. Further, this could also result in a non-uniform or inconsistent staining pattern due to differences in antigen exposure to retrieval methods.

Here we compared the 3 widely used endothelial markers Isolectin B4, CD31, and laminin for endothelial or vascular staining in PFA or glyoxal fixed free-floating brain sections. As shown in Figure. 2, we found that PFA fixed brain shows only IB4 staining while CD31 and laminin didn't show any binding. However, from our experiments and as reported before, IB4 staining is consistent in PFA fixed sections. From our search, we don't find any data showing that PFA fixation interferes with galactose residues in the cell

membrane. This inconsistent staining could be probably because of differences in sample preparation or longer tissue storage. Interestingly in glyoxal fixed brain sections, all three antibodies show consistent staining of endothelial cells/vessels.

## 3.3 Glyoxal fixation is optimal for higher quality blood-brain barrier/tight junction protein immunostaining.

The BBB restricts the movement of molecules between the blood and brain thereby maintaining cerebral homeostasis and proper neuronal function. BBB is composed of brain endothelial cells, microglia, pericytes, and a basement membrane (comprised of e.g. type IV collagen, laminin, and fibronectin), surrounded by astrocyte end-feet ensheathing (27). Leaky BBB is a serious concern in various CNS disorders like stroke, brain tumors, and neurodegenerative disorders (28). A high-resolution reliable staining method is warranted for detailed knowledge of BBB proteins, their interactions, and modifications as well as their distribution that maintains BBB integrity during normal physiology or disease.

Endothelial cells contribute to the core of BBB (29-32) . The barrier properties of these brain endothelial cells notably depend on tight junctions (TJ) between adjacent cells: TJs are dynamic structures consisting of several transmembranes and membrane-associated cytoplasmic proteins (33). Moreover, several studies have reported leaky BBB is associated with the loss of integrity in TJs (34). Here, we tested the efficacy of glyoxal fixation in staining the BBB proteins including tight junctions (TJs), transporter, and adherence junction.  We tested the effectiveness of PFA or glyoxal fixation in visualizing three significant TJ proteins, ZO-1, Occludin, and Claudin-5 (35). None of these three antibodies showed specific binding in the PFA fixed brain sections (results not shown). However, for the glyoxal-fixed brain sections, continuous filaments of TJ proteins, ZO-1, occludin, and Claudin-5 are more easily detected (Fig.3 ). This analysis suggests that the immunostainings performed after glyoxal fixation more readily allow the identification of TJ proteins. Moreover, we didn’t observe any difference with or without the use of blocking buffer indicating there is very less non-specific binding during this staining. We also tested the immunostaining of adherence junction protein VE-cadherin (36). As reported with the TJ proteins, VE-cadherin shows strong staining in the glyoxal fixed brain with no significant staining in PFA fixed samples. Further, we compared the immunostaining for a BBB transporter Aquaporin-4 (37) and found strong vascular-specific staining for both PFA and glyoxal-fixed brain samples (Figure. 4).

A possible explanation for the TJ and adherence junction proteins are not visualized in the PFA-fixed samples could be the after-effects of high crosslinking and shrinking that mask the antigen. Interestingly, AQP4 staining was visualized in both PFA and glyoxal consistently. This can be related to the location of this protein. AQP4 is a water channel protein and is expressed in brain perivascular astrocyte processes (38).  Together, direct exposure of endothelial cells to PFA while perfusion probably leads to vascular stiffness/shrinkage and masking endothelial cells proteins while this is not the case during glyoxal fixation where the brain is perfused with PBS and dissected into glyoxal solution.

# 3.4 Efficacy of PFA or glyoxal fixation in pericyte staining.

Having proved that glyoxal is an effective fixative than PFA for vascular staining, we advanced to investigate its efficiency in staining pericytes. Pericytes were found embedded within the basement membrane, both on straight sections and at branch points of capillaries, with projections extending from the soma to wrap around the underlying vessel. In recent times, various vascular functions of pericytes have been recognized including regulation of cerebral blood flow, maintenance of the blood-brain barrier (BBB), and control of vascular development and angiogenesis (39).

We used two commonly used pericyte markers NG2 proteoglycan and PDGFRB (40), for the immunohistochemical staining for pericytes. Laminin has been used as a vessel marker. When investigated using fluorescence microscopy PFA fixed brain didn't show any staining for pericyte markers NG2 and PDGFRB (results not shown), while in glyoxal fixed brain shows uniform staining for pericyte markers along the vessels. We observed that NG2 immunohistochemistry showed flat staining around the vessels. Consistent with its receptor nature  PDGFRB staining was observed as more specific, spotty, and sporadic (Figure. 4)

# 3.6 PFA vs glyoxal pros and cons

| **Comparison PFA vs Glyoxal fixation** | | |
|---|---|---|
| **Fixative** | **PFA** | **Glyoxal** |
| Toxicity | Toxic to handlers (41) | Less toxic (18) |
| Preparation | Time-consuming | Easy to prepare |
| Fixation procedure | Intra cardiac perfusion of PFA | Directly putting the tissue in glyoxal |
| The time needed for fixation | Overnight at 4°C. | Overnight at RT. |
| Storage of fixed tissue | 4°C. | 4°C. |
| Handling sections | Easy to handle | Brittle in nature, utmost care is needed |
| Antigen retrieval | Required | Not necessary |
| Blocking | Required | Not necessary |
| Vascular Staining quality | Not always satisfactory | High-resolution staining with all the tested vascular stainings. |
| Cost | Less expensive | Moderately Expensive |

In summary,  with support of the above evidence, glyoxal represents the best alternative for 4% PFA as it is more efficient, specific, easy to fix, and highly valuable for illustrating vascular proteins at a molecular level. This methodology is significant since the discovery of BBB therapeutics highly depended on visualizing or analyzing the molecular components of BBB.

# Declarations

### Ethics approval and consent to participate

All animal procedures were carried out under the oversight of the animal care and use committee of the University of Texas Health Science Center at Houston( protocol # AWC-19-0120 ) and in strict compliance with National Institutes of Health guidelines.

### Availability of data and materials

Data will be shared upon reasonable request.

### Competing interests

The authors declare there is no conflict of interest for this manuscript.

### Consent for publication.

Not Applicable


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### Authors contribution

PKT and ST designed and performed experiments and analyzed data. JS runs preliminary vascular stainings. PKT and ST wrote the manuscript. All authors commented and contribute to the manuscript. PKT supervised the study. All authors read and approved the final manuscript.


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# Figures

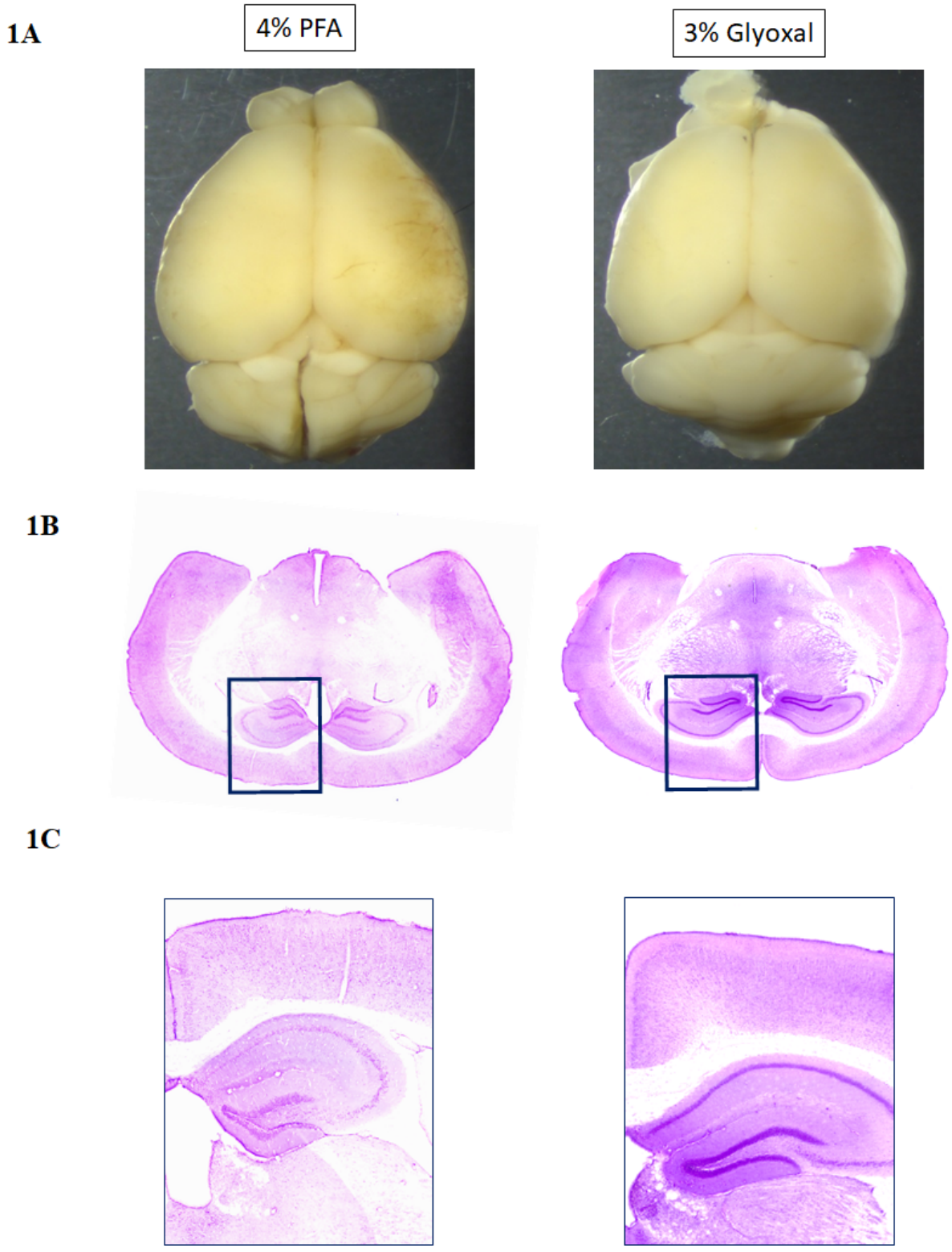
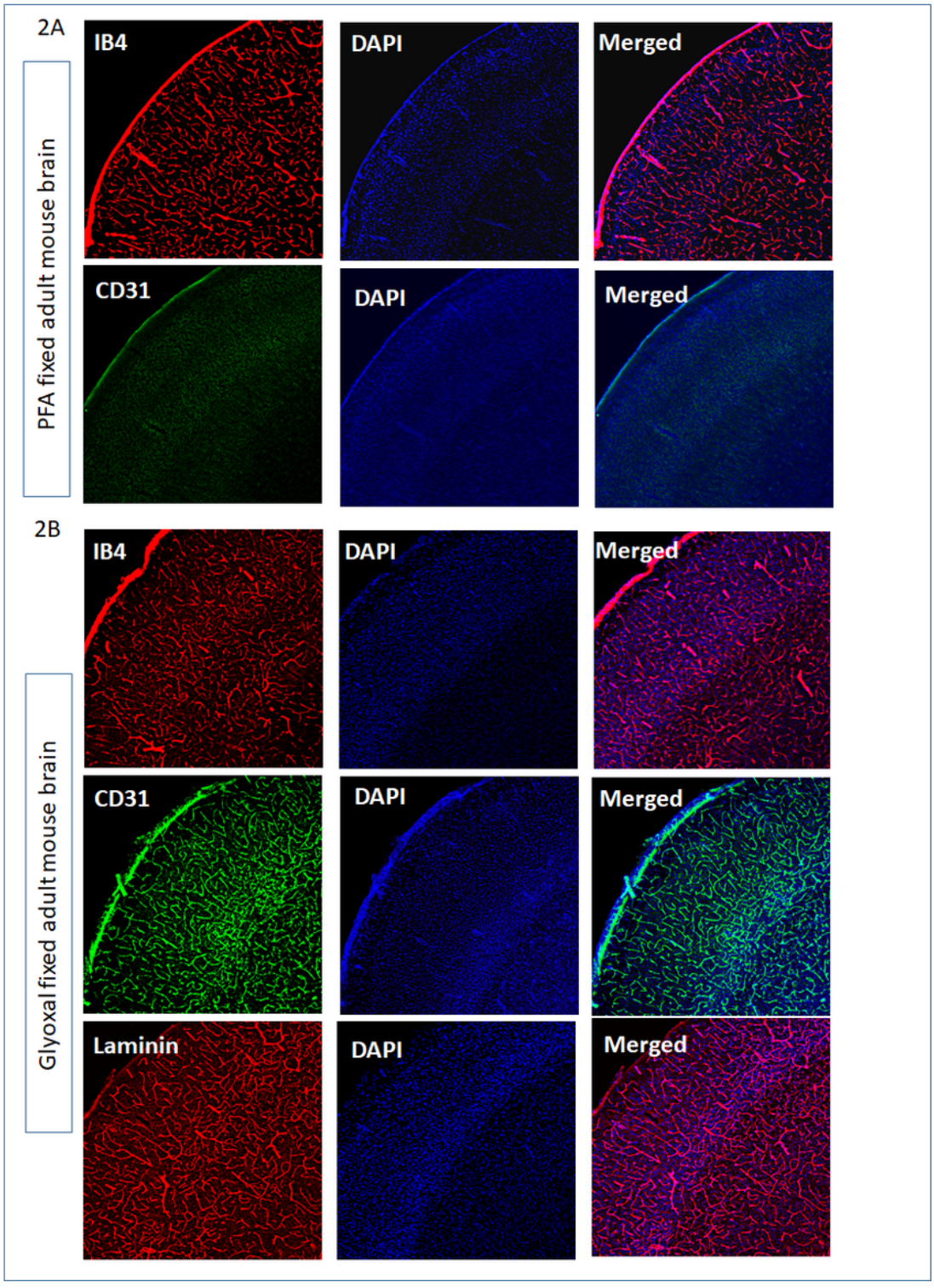



**Figure 1**

**PFA versus Glyoxal fixed mouse brain: Gross Anatomy and H&E staining.**

**1A**: Comparison of whole mouse brain after 4% PFA and 3% glyoxal fixation (5x). **1B and 1C**: The coronal plane slices from PFA or glyoxal fixed brain sections after Hematoxylin and Eosin staining (5x).

Figure-2

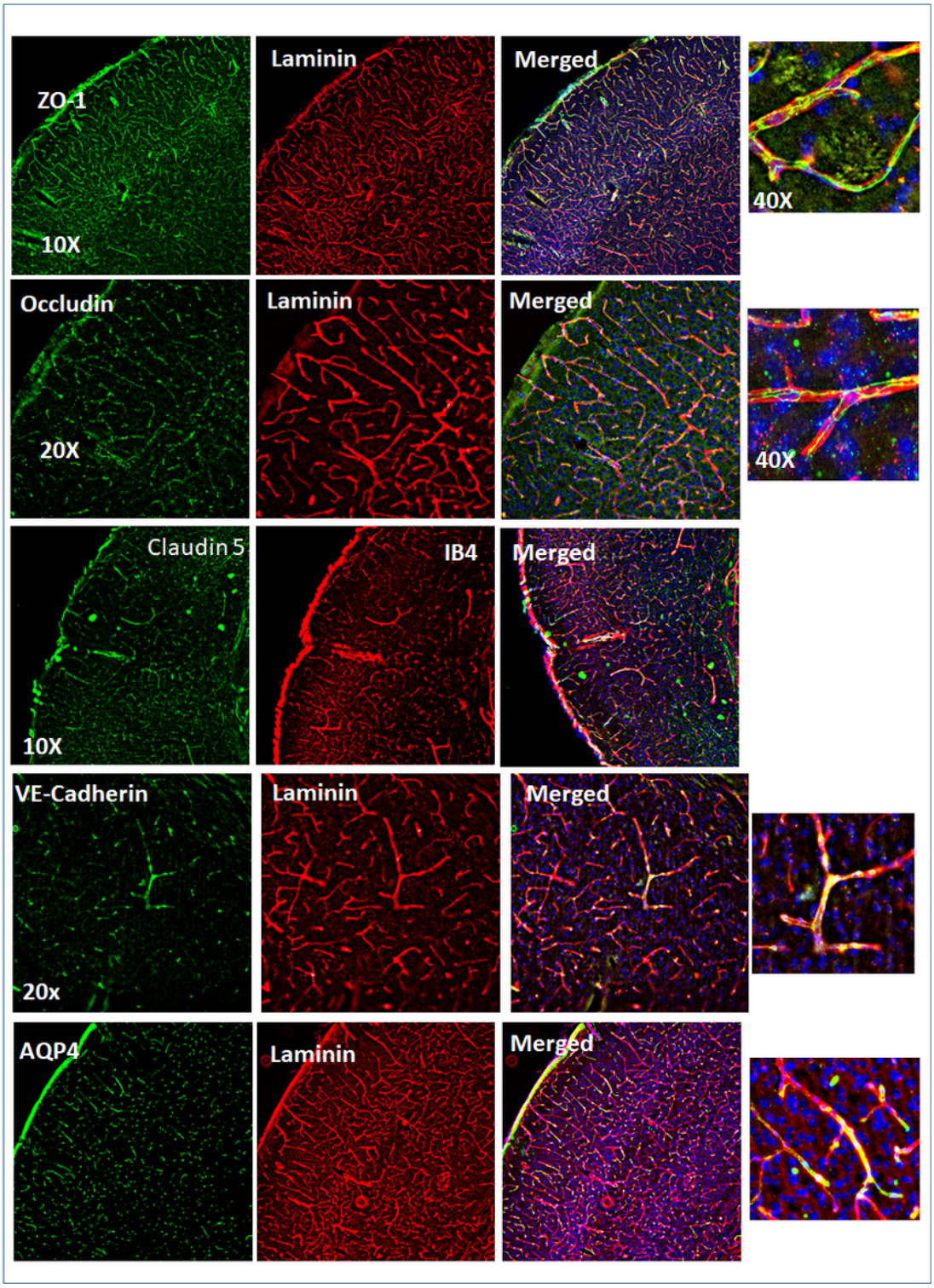



## Figure 2

**Immunostaining of commonly used antibodies in vascular staining comparison between 4% PFA and 3% glyoxal fixation. 2A.** 35 microns coronal plane slices of PFA fixed brain after IB4 (red) and CD 31(green)

staining and nuclei were detected by DAPI nuclear stain. **2B**. The coronal plane slices of glyoxal fixed brain after IB4(red), CD 31 (green), and laminin staining (red), nuclei were detected by DAPI nuclear stain.

Figure-3




## Figure 3

**Tight junction and adherence junction staining in 3% glyoxal fixed brain .** 35 microns coronal sections from glyoxal fixed brain samples stained with ZO-1, Occludin, Claudin 5, VE-Cadherin, and Aquaporin (AQP4) antibodies as tight junction/BBB markers and laminin or IB4 was used for common vascular staining.

Figure-4

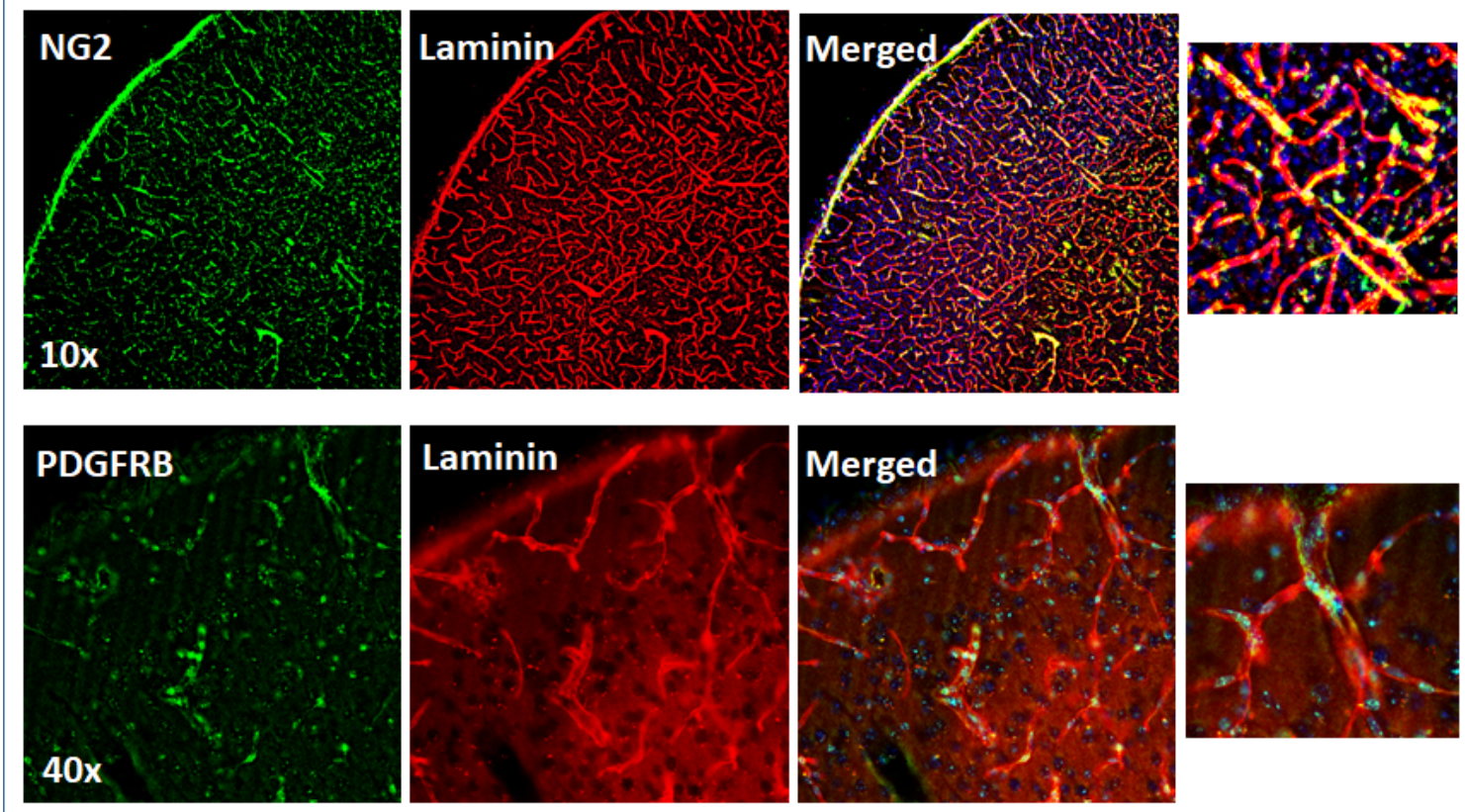



**Figure 4**

**Pericyte staining in 3% glyoxal fixed brain.** Pericyte staining of brain coronal plane sections after glyoxal fixation. Pericytes in brain sections were detected by common pericyte markers, NG2 and PDGFRB, and laminin was used for vascular staining.