**Advances in Biochemistry**
2022; 10(2): 59-70
http://www.sciencepublishinggroup.com/j/ab
doi: 10.11648/j.ab.20221002.14
ISSN: 2329-0870 (Print); ISSN: 2329-0862 (Online)

# Comparison of Long-Term Fixation Effects on Tissues Stored in Glyoxal and Formaldehyde as Evaluated by Special Stains and Immunohistochemistry

**Sheila Criswell[1, *], Kristina Zwerg[2], Cynthia Lazar[2], Caitlin Richmond[1], Chelsea Bridges[1]**

[1]Department of Diagnostic and Health Sciences, University of Tennessee Health Science Center, Memphis, USA

[2]Department of Pathology, Methodist University Hospital, Memphis, USA

## Email address:

scriswel@uthsc.edu (S. Criswell)

[*]Corresponding author

## To cite this article:

Sheila Criswell, Kristina Zwerg, Cynthia Lazar, Caitlin Richmond, Chelsea Bridges. Comparison of Long-Term Fixation Effects on Tissues Stored in Glyoxal and Formaldehyde as Evaluated by Special Stains and Immunohistochemistry. *Advances in Biochemistry*. Vol. 10, No. 2, 2022, pp. 59-70. doi: 10.11648/j.ab.20221002.14

**Received**: May 6, 2022; **Accepted**: May 23, 2022; **Published**: May 31, 2022

**Abstract:** Glyoxal solutions are safer fixatives as compared to formalin in the anatomic pathology laboratory. While long-term effects of tissue stored in formalin are well-documented, studies of tissues fixed long-term in glyoxal are few. Using human autopsy tissues, 4 glyoxal solutions, and neutral buffered formalin, the effects of storage of tissues in fixatives for 8 hours, 1 month, and 4 months were compared. Special stain methods to include hematoxylin and eosin, melanin bleaching using either 10% hydrogen peroxide or potassium permanganate followed by oxalic acid, Verhoeff van Gieson, reticulin, Fontana Masson, mucicarmine, colloidal iron, alcian blue, trichrome, and Wright stain, as well as immunohistochemistry labeling of 18 antibodies were conducted and compared. Tissues fixed in glyoxal solutions exhibited nearly identical staining properties when compared with each other for all lengths of fixation. When fixed for 8 hours, they also produced the same results as tissues fixed in formalin. Although glyoxal-fixed tissues stored for 1 – 4 months in fixatives mimicked staining in formalin-fixed tissues for most special stain techniques, they retained adequate antigenic labeling for only 78% of the antibodies evaluated. Consequently, for special stain applications, tissues fixed in glyoxal for both short and long term yielded results comparable to those fixed in formalin. However, careful validation of specific antibody clones should be undertaken when conducting immunohistochemistry evaluations on tissues stored long-term in glyoxal fixatives.

**Keywords:** Fixation, Fixative, Formalin, Glyoxal, Immunohistochemistry, Long-Term, Special Stains

## 1. Introduction

"Long-term fixation" is a phrase applied with subjectivity to the duration an organismal tissue is immersed in a fixative solution for the purpose of preserving the tissue for subsequent examination. Chemical fixatives prevent the breakdown and loss of tissue elements and allow for performance of multiple assessments at a later date. In the routine anatomic pathology lab, surgically-removed tissues are immediately placed into a fixative solution, most commonly 10% neutral buffered formalin (NBF), for 6 – 72 hours before undergoing processing through a series of increasing concentrations of alcohol, several changes of a clearing agent, and finally molten paraffin before the tissues are embedded into paraffin blocks for further testing. The need to return pathology results to clinicians quickly to treat patients more effectively has driven laboratory managers to reduce turnaround time to roughly 24 hours for tissue processing and routine hematoxylin and eosin (H&E) staining. Therefore, "long-term fixation" may realistically suggest chemical fixation for any length of time greater than 72 hours. Subsequent testing with special stains, immunohistochemistry (IHC) evaluations, and molecular examinations frequently add to the turnaround time, but are necessary assessments to optimally guide patient treatment.

Per the College of American Pathologists (CAP) accreditation body for clinical and anatomic pathology laboratories in the United States, surplus anatomic pathology

tissues not submitted for pathologist review or further testing must be retained for a minimum of 2 weeks following release of the final pathology report before discard. The 2-week buffer period allows for all parties participating in patient care ample opportunity to review the pathology report and request further testing should it be needed. In medico-legal cases, autopsy tissues may be retained for months while brains used in teaching or research may be kept for years. In the past, various investigators have defined long-term fixation in very subjective terms including up to 12 days [1], up to 30 days [2-5], 7 – 154 days [6], up to 1 year [7-9], 3-12 years [10], 6 months – 14 years [11], 34 – 47 years [12].

The actions of formalin and glutaraldehyde as preservatives on fresh tissues are to form cross links both intra- and intermolecularly [13]. The cross links, formed by the addition of the fixative molecules to the tissue elements (glutaraldehyde more rapidly than formaldehyde) not only have the advantageous property of firming the tissues, but also have the disadvantage of altering the shape of molecules and physically impeding recognition of antibodies for, and attachment to, their native antigenic sites [13]. The action of glyoxal fixatives in cross linking tissue molecules is significantly reduced compared with those of formalin and glutaraldehyde [14] which may reduce the need for antigen retrieval steps prior to IHC procedures [14, 15].

Due to the carcinogenic nature of formalin, some countries have mandated reduction in its use [9]. Glyoxal, however, due to its affinity for the hydrated state, releases few or no toxic vapors [14] and can be used without the special ventilation or vapor filtration provided by a grossing hood. Glyoxal can be purchased in its pure concentrated form, but, as a commercial fixative, is mixed in a buffering solution with an accelerator [14]. Accelerators most commonly used are alcohols per the Safety Data Sheets supplied by the manufacturers of commercially available glyoxal solutions. However, the specific alcohol added and concentration thereof varies somewhat among the commercial fixatives and are proprietorial information. Putatively, however, the amount of alcohol added does not exceed that of 20% so that the protein conformation is not unduly affected by the irreversible structural changes produced by alcohol fixation alone, and these commercial solutions are not considered flammable by OSHA standards [14].

Several studies have verified that hematoxylin and eosin (H&E) and most special stains work equally well on glyoxal-fixed tissues as compared to those fixed in NFB [14, 16-18]. Immunohistochemical studies, however, have produced mixed results [5, 15-17, 19-22]. The goal of the current study is to evaluate H&E, special stains, and IHC markers in glyoxal-fixed tissues which have been stored in fixative for 1 month and 4 months as they compare with tissues processed immediately after 8 hours fixation in glyoxal and formalin fixatives.

# 2. Materials and Methods

### *2.1. Tissues*

This study was approved under the University of Tennessee Internal Review Board (IRB) number 21-08027-NHSR. Fresh unfixed tissues including skin, spleen, tongue, liver, breast, small intestine, and lung were obtained from two autopsy patients. Within 24 hours of ischemic time, tissues were grossed into 3 mm slices and immersed into 5 different fixative solutions. Solutions included commercially-prepared 3.7% w/v neutral buffered formaldehyde (NBF), and 4 glyoxal-based solutions: an in-house prepared glyoxal/ethanol solution (GE), Glyo-Fixx™ (GF), Prefer (PF), and Preserve (PS) (Table 1). The GE fixative was produced by diluting 50 ml 40% aq. glyoxal with 75 ml ethanol and 375 ml 0.1 M sodium acetate buffer. The sodium acetate buffer was prepared by adding 7.7 g anhydrous sodium acetate (S210, Fisher Scientific, Belgium) to de-ionized water ($DIH_2O$) for a final volume of 1 liter and then adding 20 drops of glacial acetic acid (1101, EK Industries, USA).

***Table 1.*** *Fixatives evaluated included neutral buffered formalin and 4 different glyoxal-based fixatives.*

| Fixative | Catalog Number | Manufacturer | pH | Abbreviation |
|---|---|---|---|---|
| 3.7% neutral buffered formalin | SF100-4 | Fisher Chemical, USA | 7.0 | NBF |
| 4% glyoxal / 15% ethanol in sodium acetate buffer | Glyoxal: A16144<br>Ethanol: 111000200 | Alfa Aesar/ Thermo Fisher Pharmco, USA | 5.9 | GE |
| Glyo-Fixx™ | 6764262 | Shandon, USA | 4.3 | GF |
| Prefer | NC9053360 | Anatech, Ltd, USA | 4.3 | PF |
| Preserve | LP2808 | EBSciences, USA | 4.3 | PS |

For each fresh autopsy specimen, 3 duplicate sets of tissues were placed into the 5 fixative solutions. One set was allowed to fix for 8 hours, rinsed in tap water for 2 minutes, and then processed on a closed tissue processor using increasing concentrations of alcohol, 3 changes of xylene, and 4 changes of paraffin. The second set of tissues was allowed to remain in fixative solution for 1 month before undergoing the same processing schedule as indicated above, while the third set of tissues was held in fixative for 4 months before undergoing processing. In total, for each organ, there were 15 fixation conditions: 5 fixative solutions over 3 time periods. Once processed and embedded, 4 µm sections were stained with hematoxylin and eosin (H&E) to evaluate overall morphology and to select regions of interest for subsequent staining techniques. For all autopsy tissues, 2 mm – 4 mm cores were selected from each tissue block and were assembled into tissue microarrays (TMA) so that all fixative conditions were represented in a single tissue block. Array sections were mounted onto plus-charged slides and heated to 40°C overnight.

### *2.2. Deparaffinization*

All H&E, special stains, and IHC protocols began with tissue deparaffinization consisting of immersion of tissue sections into 3 changes of xylene for 1 minute each, 2 changes of 100% ethanol for 10 seconds each with agitation, 1 change of 95% ethanol for 10 seconds with agitation, and then finally to deionized water until the staining protocol began.

### *2.3. Special Stain Techniques*

Special stains performed included 2 melanin bleaching protocols (Tables 2 and 3) followed by H&E (Table 4), modified trichrome (Table 5), Verhoeff van Gieson (Table 6), mucicarmine (Table 7), alcian blue (Table 8), colloidal iron (Table 9), reticulin (Table 10), Wright (Table 11), and Fontana Masson (Table 12). Additionally, an alkaline alcohol solution was made by adding 3 ml of 3% aqueous sodium hydroxide (S1920, Poly Scientific R&D, USA) to 50 ml absolute ethanol for a final pH of 9.0. This solution was used to remove fixative pigment in tissue sections. Modified H&E protocols using standard H&E reagents, basic fuchsine, picrosirius red, and safranine O were evaluated in the attempt to improve nuclear/cytoplasmic contrast (Tables 13-16). After completion of each staining protocol (excluding Wright stain), tissue sections were immersed into 95% ethanol for 10 seconds with agitation, 3 changes of absolute ethanol for 10 seconds each with agitation, and 3 changes of xylene for 1 minute each prior to being coverslipped with a resinous mounting medium.

***Table 2.*** *Melanin Bleaching Protocol 1.*

| Step | Reagent | Time |
|---|---|---|
| 1 | Working bleaching solution made by adding 500 µl Formamide (BP228, Fisher Scientific, USA) to 4.5 ml 10% H2O2 (HPF2006, Lab Alley, USA) | 15 minutes under bright light using 43-watt bulb in a desk lamp |
| 2 | Running tap water | 1 minute |
| 3 | H&E protocol (see Table 4) | |

***Table 3.*** *Melanin Bleaching Protocol 2.*

| Step | Reagent | Time |
|---|---|---|
| 1 | 1.0% potassium permanganate (AHP0726, American MasterTech Scientific, Inc., USA) | 5 minutes |
| 2 | Running tap water | 1 minute |
| 3 | Oxalic acid, 98% anhydrous (186432500, ACROS Organics, USA) | 30 seconds with agitation |
| 4 | Running tap water | 1 minute |
| 5 | H&E protocol (see Table 4) | |

***Table 4.*** *Hematoxylin and Eosin (H&E).*

| Step | Reagent | Time |
|---|---|---|
| 1 | SelecTech Hematoxylin 560 MX (3801575, Leica Surgipath, VIC) | 5 minutes |
| 2 | Deionized water | 1 minute |
| 3 | Acid alcohol (0.5% concentrated HCl in 50% ethanol) | 5 seconds with agitation |
| 4 | Deionized water | 1 minute |
| 5 | Vintage bluing (SL102, Stat Lab, USA) | 30 seconds |
| 6 | Deionized water | 1 minute |
| 7 | 95% ethanol | 10 seconds with agitation |
| 8 | Select Eosin (SL406, Stat Lab, USA) | 2 minutes, 15 seconds |

***Table 5.*** *Modified Trichrome.*

| Step | Reagent | Time |
|---|---|---|
| 1 | Bouin Fixative (S129, Poly Scientific R&D Corporation, USA) | 15 minutes at 60°C |
| 2 | Running tap water | 5 minutes |
| 3 | Biebrich Scarlet Acid Fuchsin (AHB0443, American MasterTech, USA) | 5 minutes |
| 4 | Running tap water | 1 minute |
| 5 | Phosphomolybdic acid phosphotungstic acid (AHP0543, American MasterTech, USA) | 10 minutes |
| 6 | Aniline blue (AHA0543, American MasterTech, USA) | 10 minutes |
| 7 | Running tap water | 1 minute |

***Table 6.*** *Verhoeff van Gieson.*

| Step | Reagent | Time |
|---|---|---|
| 1 | Verhoeff's working hematoxylin prepared by mixing 15 ml 10% hematoxylin (525347, Fisher Science Education, USA) in absolute ethanol, 6 ml 10% ferric chloride (AHF0143, American MasterTech, USA), and 6 ml Lugol's iodine Working Solution (s234A, Poly Scientific R&D Corporation, USA) | 30 minutes |
| 2 | Running tap water | 1 minute |
| 3 | 2% ferric chloride (S180, Poly Scientific R&D Corporation, USA) | Approximately 20 seconds with agitation until microscopically differentiated |
| 4 | Running tap water | 30 seconds |

| Step | Reagent | Time |
|---|---|---|
| 5 | 5% sodium thiosulfate (26374-05, Electron Microscopy Sciences, USA) | 1 minute |
| 6 | Running tap water | 30 seconds |
| 7 | Van Gieson's Solution (26374-06, Electron Microscopy Sciences, USA) | 5 minutes |
| 8 | Running tap water | 10 seconds |

***Table 7.*** *Mucicarmine.*

| Step | Reagent | Time |
|---|---|---|
| 1 | Iron hematoxylin prepared by adding 10 ml 2% ferric chloride (S180, Poly Scientific R&D Corporation, USA) to 2.5% hematoxylin (525347, Fisher Science Education, USA) in absolute ethanol | 10 minutes |
| 2 | Running tap water | 3 minutes |
| 3 | Working mucicarmine solution made by diluting 1 part Mucicarmine Stock Stain, Mayer (1250°C, Newcomer Supply, USA) with 3 parts tap water | 30 minutes at 60°C |
| 4 | Running tap water | 1 minute |
| 5 | Metanil Yellow 0.25% aqueous (S239, Poly Scientific R&D Corporation, USA) | 1 minute |
| 6 | Running water | 30 seconds |

***Table 8.*** *Alcian Blue.*

| Step | Reagent | Time |
|---|---|---|
| 1 | 3% acetic acid (Acetic Acid, Glacial, 1101, Eki-chem, USA) | 5 minutes |
| 2 | Alcian Blue 1% in 3% Acetic Acid pH 2.5 (S111A, Poly Scientific R&D Corporation, USA) | 30 minutes |
| 3 | 3% acetic acid | 10 seconds with agitation |
| 4 | Running tap water | 10 minutes |
| 5 | Nuclear Fast Red (STNFRLT, American MasterTech Scientific, Inc., USA) | 5 minutes |
| 6 | Running tap water | 1 minute |

***Table 9.*** *Colloidal Iron.*

| Step | Reagent | Time |
|---|---|---|
| 1 | 12% acetic acid (Acetic Acid, Glacial, 1101, Eki-chem, USA) | 1 minute |
| 2 | Colloidal iron working solution made by mixing 5 ml colloidal iron stock solution (10365B, Newcomer Supply, USA) with 15 ml 40% acetic acid | 30 minutes |
| 3 | 12% acetic acid | 3 minutes |
| 4 | 12% acetic acid | 3 minutes |
| 5 | 12% acetic acid | 3 minutes |
| 6 | Developer solution made by adding equal parts 5% potassium ferrocyanide (Potassium ferrocyanide trihydrate, 424135000, ACROS Organics, Belgium) to 5% HCl (A144-212, Fisher Chemical, USA) | 20 minutes |
| 7 | Running tap water | 5 minutes |
| 8 | Van Gieson's Solution (26374-06, Electron Microscopy Sciences, USA) | 5 minutes |
| 9 | Running tap water | 10 seconds |

***Table 10.*** *Reticulin.*

| Step | Reagent | Time |
|---|---|---|
| 1 | 1.0% potassium permanganate (AHP0726, American MasterTech Scientific, Inc., USA) | 5 minutes |
| 2 | Running deionized water | 1 minute |
| 3 | 3% potassium metabisulfite (KC3921, American MasterTech, Lodi, USA) | 1 minute |
| 4 | Running deionized water | 1 minute |
| 5 | 3% Ferric ammonium sulfate (KC3933, American MasterTech, USA) | 10 minutes |
| 6 | Running deionized water | 1 minute |
| 7 | Working silver solution made by combining 500 µl Type 1 silver solution (T15-03, American MasterTech, USA) with 3 ml deionized water, then adding concentrated ammonium hydroxide (A669, Fisher Scientific, USA) until precipitate dissolves. Then, 1.5 ml 3% sodium hydroxide (AH50626, American MasterTech, USA) is added and redissolved using concentrated ammonium hydroxide. Lastly, 5 ml of deionized water was added and the solution mixed. | 2 minutes |
| 8 | Running deionized water | 1 minute |
| 9 | 20% aqueous formalin | 1 minute |
| 10 | Running deionized water | 2 minutes |
| 11 | 0.2% gold chloride (AHGCH, American MasterTech, USA) | 5 minutes |
| 12 | Running deionized water | 1 minute |
| 13 | 5% sodium thiosulfate (26374-05, Electron Microscopy Sciences, USA) | 1.5 minutes |
| 14 | Running deionized water | 1 minute |
| 15 | Nuclear Fast Red (STNFRLT, American MasterTech Scientific, Inc., USA) | 5 minutes |
| 16 | Running deionized water | 1 minute |

***Table 11.*** *Wright.*

| Step | Reagent | Time |
|---|---|---|
| 1 | Methanol (CY324, Cardinal Health, USA) | 1 minute |
| 2 | Coulter TruColor Wright stain (7502429-DE, Beckman Coulter, Inc, USA) | 4 minutes |
| 3 | Working Wright buffer made by mixing 1 part Wright stain with 3 parts Coulter TruColor Wright Stain buffer (75024370-CC, Beckman Coulter, Inc. USA) | 3 minutes |
| 4 | Running tap water | 1 minute |
| 5 | Slides were stood on end and allowed to dry before briefly immersing in xylene prior to coverslipping | |

***Table 12.*** *Fontana Masson.*

| Step | Reagent | Time |
|---|---|---|
| 1 | Ammoniacal Silver Nitrate Solution Gomoris (s114, Poly Scientific R&D Corporation, USA) | 10 minutes at 55C |
| 2 | Running deionized water | 1 minute |
| 3 | 0.2% gold chloride (AHGCH0.2, American MasterTech, USA) | 1 minute |
| 4 | Running deionized water | 1 minute |
| 5 | 5% sodium thiosulfate (26374-05, Electron Microscopy Sciences, USA) | 2 minutes |
| 6 | Running deionized water | 1 minute |
| 7 | Nuclear Fast Red (STNFRLT, American MasterTech Scientific, Inc., USA) | 5 minutes |
| 8 | Running deionized water | 1 minute |

***Table 13.*** *Modified Hematoxylin and Eosin (H&E).*

| Step | Reagent | Time |
|---|---|---|
| 1 | SelecTech Hematoxylin 560 MX (3801575, Leica Surgipath, VIC) | 1 minute |
| 2 | Deionized water | 1 minute |
| 3 | Acid alcohol (0.5% concentrated HCl in 50% ethanol) | 5 seconds with agitation |
| 4 | Deionized water | 1 minute |
| 5 | Vintage bluing (SL102, Stat Lab, USA) | 30 seconds |
| 6 | Deionized water | 1 minute |
| 7 | 95% ethanol | 10 seconds with agitation |
| 8 | Select Eosin (SL406, Stat Lab, USA) | 6 minutes |

***Table 14.*** *Hematoxylin and Basic Fuchsine.*

| Step | Reagent | Time |
|---|---|---|
| 1 | SelecTech Hematoxylin 560 MX (3801575, Leica Surgipath, VIC) | 5 minutes |
| 2 | Deionized water | 1 minute |
| 3 | Acid alcohol (0.5% concentrated HCl in 50% ethanol) | 5 seconds with agitation |
| 4 | Deionized water | 1 minute |
| 5 | Vintage bluing (SL102, Stat Lab, USA) | 30 seconds |
| 6 | Deionized water | 1 minute |
| 7 | 0.13% basic fuchsin (220, EM Diagnostic Systems, Inc., USA) | 30 seconds |

***Table 15.*** *Hematoxylin and Picrosirius Red.*

| Step | Reagent | Time |
|---|---|---|
| 1 | SelecTech Hematoxylin 560 MX (3801575, Leica Surgipath, VIC) | 5 minutes |
| 2 | Deionized water | 1 minute |
| 3 | Acid alcohol (0.5% concentrated HCl in 50% ethanol) | 5 seconds with agitation |
| 4 | Deionized water | 1 minute |
| 5 | Vintage bluing (SL102, Stat Lab, USA) | 30 seconds |
| 6 | Deionized water | 1 minute |
| 7 | Picrosirius solution made by adding 0.5 g Sirius Red (Direct Red 80, 22913, Chem-Impex International, Inc., USA) to 500 ml saturated picric acid, aqueous (5860-32. Ricca Chemical Company, USA) | 2 minutes, 15 seconds |

***Table 16.*** *Hematoxylin and Safranine O.*

| Step | Reagent | Time |
|---|---|---|
| 1 | SelecTech Hematoxylin 560 MX (3801575, Leica Surgipath, VIC) | 5 minutes |
| 2 | Deionized water | 1 minute |
| 3 | Acid alcohol (0.5% concentrated HCl in 50% ethanol) | 5 seconds with agitation |
| 4 | Deionized water | 1 minute |
| 5 | Vintage bluing (SL102, Stat Lab, USA) | 30 seconds |
| 6 | Deionized water | 1 minute |
| 7 | 1% Safranin O (STSF0100, American MasterTech, USA), diluted 1:16 with water | 1 minute |

### *2.4. Immunohistochemistry (IHC) Labeling*

Tissue sections were deparaffinized to deionized water as indicated above and then were placed into plastic coplin jars with one of the following antigen retrieval solutions: high pH, low pH, or Tris-HCl (Table 17). Antigenic sites were decloaked 5 minutes at 120°C using a Biocare Decloaking Chamber (Biocare Medical, USA). After the retrieval solutions cooled to room temperature (RT), sections were rinsed in 3 changes of deionized water and then placed in 1X Envision™ Flex Wash buffer (TBS) (DM831, Dako, USA). All IHC steps were performed manually in a humidity chamber at RT. Primary antibodies used in IHC can be found in Table 18. Hydrophobic barriers were drawn around the tissue sections and sections were not allowed to dry during the IHC procedure. Positive and negative controls were examined with all IHC procedures and were deemed acceptable before analysis of TMAs. The brown IHC kit was Bond™ Polymer Refine Detection Kit, lot 66800 (DS9800, Leica Biosystems, USA) (Table 19), while the red kit was Bond™ Polymer Refine Red Detection Kit, lot 65033 (DS9390, Leica Biosystems) (Table 20).

***Table 17.*** *Antigen retrieval solutions.*

| Retrieval Solution | Manufacturer Information | Formulation | pH | Decloaking Device* |
|---|---|---|---|---|
| EnVision FLEX Target Retrieval Solution High pH 50x | K800421-2, Dako/Agilent | Diluted 1:50 to 1X with $DIH_2O$ | 8.5 | 5 minutes at 120°C |
| IHC Antigen Retrieval Solution (10x) | 00-4955-58, Invitrogen, USA | Diluted 1:10 to 1X with DIH2O | 6.0 | 5 minutes at 120°C |
| 1.0 M Tris HCl buffer | RGE3363, Fisher Scientific, USA | Diluted 1:10 with $DIH_2O$ to 0.1 M | 8.5 | 5 minutes at 120°C |

*Biocare Decloaking Chamber (Biocare Medical, USA).

***Table 18.*** *List of antibodies tested.*

| Antibody | Manufacturer | Catalog Number | RRID | Lot | Antibody Dilution |
|---|---|---|---|---|---|
| Actin, muscle sp. | Cell Marque | HHF35 | AB_1157932 | 1518802E | 1:1000 |
| AE1/AE3 | Leica Novacastra | PA0094 | NA | 68111 | RTU |
| CD3 | Leica Novacastra | PA0122 | NA | 65275 | RTU |
| CD15 | Leica Novacastra | PA0473 | NA | 62049 | RTU |
| CD20 | Leica Novacastra | PA0200 | NA | 69259 | RTU |
| CD34 | Cell Marque | 134M-16 | AB_1159227 | 0000058192 | 1:100 |
| CD34 | Leica Novacastra | PA0212 | NA | 65080 | RTU |
| CD163 | Cell Marque | 163M-16 | AB_1159122 | 0000037062 | 1:500 |
| CK 5/6 | Ventana/Roche | 790-4554 | NA | Y28255 | RTU |
| CK 7 | Leica Novacastra | PA0138 | NA | 64915 | RTU |
| CK 8/18 | Ventana/Roche | 760-4344 | NA | V0002101 | RTU |
| Desmin | Leica Novacastra | PA0032 | NA | 63795 | RTU |
| Ki-67 | Ventana/Roche | 790-4286 | AB_2631262 | G23748 | RTU |
| Pankeratin | Epredia | MS-343-P | NA | 343P2002E | 1:200 |
| S100 | Dako/Agilent | Z0311 | AB_10013383 | 20014502 | 1:4000 |
| S100 | Leica Novacastra | PA0031 | NA | 66838 | RTU |
| Vimentin | Dako/Agilent | M7020 | AB_2304493 | 20024468 | 1:1000 |
| Vimentin | Leica Novacastra | PA0640 | NA | 63200 | RTU |

RTU = Ready to use (no dilution needed). NA = None available.

***Table 19.*** *Brown IHC Protocol. Bond™ Polymer Refine Detection Kit, lot 66800 (DS9800, Leica Biosystems, USA).*

| Step | Reagent | Time |
|---|---|---|
| 1 | Kit reagent Peroxidase Block | 5 minutes |
| 2 | Tris buffered saline (TBS) | Holding slides at an incline, rinse 3 times with squirt bottle |
| 3 | Primary antibody | 20 minutes |
| 4 | TBS | Holding slides at an incline, rinse 3 times with squirt bottle |
| 5 | Kit Post Primary reagent | 8 minutes |
| 6 | TBS | 2 minutes |
| 7 | TBS | 2 minutes |
| 8 | TBS | 2 minutes |
| 9 | Kit Polymer | 8 minutes |
| 10 | TBS | 2 minutes |
| 11 | TBS | 2 minutes |
| 12 | Deionized water | Holding slides at an incline, rinse 2 times with squirt bottle |
| 13 | Brown Chromogen (Kit reagents Part 1 and Part B) | 2 minutes |
| 14 | Deionized water | 2 minutes |
| 15 | Kit Hematoxylin | 5 minutes |
| 16 | Deionized water | Holding slides at an incline, rinse 2 times with squirt bottle |
| 17 | TBS buffer | 30 seconds |

| Step | Reagent | Time |
|---|---|---|
| 18 | Deionized water | Holding slides at an incline, rinse 2 times with squirt bottle |
| 19 | After final deionized water rinse, slides were allowed to air dry. Once dry, the slides were briefly immersed in fresh xylene and were coverslipped with a resinous mounting medium. | |

***Table 20.*** *Red Detection Kit IHC. Bond™ Polymer Refine Red Detection Kit, lot 65033 (DS9390, Leica Biosystems).*

| Step | Reagent | Time |
|---|---|---|
| 1 | Primary antibody | 30 minutes |
| 2 | TBS | Holding slides at an incline, rinse 1 time with squirt bottle and then apply for 1 minute |
| 3 | Kit Post Primary AP | 20 minutes |
| 4 | TBS | 2 minutes |
| 5 | TBS | 2 minutes |
| 6 | Kit Polymer AP | 30 minutes |
| 7 | TBS | 2 minutes |
| 8 | TBS | 2 minutes |
| 9 | TBS | 5 minutes |
| 10 | TBS | 2 minutes |
| 11 | Deionized water | Holding slides at an incline, rinse 3 times with squirt bottle |
| 12 | Kit reagents Parts (A-D) | 3 minutes |
| 13 | Deionized water | Holding slides at an incline, rinse 3 times with squirt bottle |
| 14 | Kit Hematoxylin | 1 minute |
| 15 | Deionized water | Holding slides at an incline, rinse 3 times with squirt bottle |
| 16 | TBS | 30 seconds |
| 17 | Deionized water | Holding slides at an incline, rinse 3 times with squirt bottle |
| 18 | After final deionized water rinse, slides were allowed to air dry. Once dry, the slides were briefly immersed in fresh xylene and were coverslipped with a resinous mounting medium. | |

***Table 21.*** *Antigenicity retention of glyoxal-fixed tissues as labeled by antibodies.*

| Antigen | Manufacturer of Primary Antibody | Dilution of Supplied Primary Antibody | 8h fixation | 1mo fixation | 4mo fixation |
|---|---|---|---|---|---|
| Actin, muscle sp. | Cell Marque | 1:1000 | 3 | 3 | 3 |
| AE1/AE3 | Leica Novacastra | RTU | 3 | 2.5 | 2.5 |
| CD3 | Leica Novacastra | RTU | 3 | 2 | 2 |
| CD15 | Leica Novacastra | RTU | 3 | 3 | 3 |
| CD20 | Leica Novacastra | RTU | 3 | 3 | 3 |
| CD34 | Cell Marque | 1:100 | 3 | 2 | 1.5 |
| CD34 | Leica Novacastra | RTU | 3 | 3 | 2 |
| CD163 | Cell Marque | 1:500 | 3 | 3 | 3 |
| CK 5/6 | Ventana/Roche | RTU | 3 | 2.5 | 2 |
| CK 7 | Leica Novacastra | RTU | 3 | 3 | 3 |
| CK 8/18 | Ventana/Roche | RTU | 3 | 3 | 2 |
| Desmin | Leica Novacastra | RTU | 3 | 1 | 0 |
| Ki-67 | Ventana/Roche | RTU | 3 | 0 | 0 |
| Pankeratin | Epredia | 1:200 | 3 | 2.5 | 2.5 |
| S100 | Dako/Agilent | 1:4000 | 3 | 3 | 3 |
| S100 | Leica Novacastra | RTU | 3 | 3 | 3 |
| Vimentin | Dako/Agilent | 1:1000 | 3 | 1 | 0 |
| Vimentin | Leica Novacastra | RTU | 3 | 3 | 2 |

RTU = Ready to use (no dilution needed).

### *2.5. Analysis*

IHC stains were scored using a 0 – 3 system where 0 = no visible chromogen, 1 = weak chromogen labeling unacceptable for clinical diagnosis, 2 = moderate chromogen labeling, not optimal but acceptable, and 3 = optimal chromogen labeling. Half-step scores of 0.5, 1.5, and 2.5 were rarely employed when the quantity of labeled cells was lower than normal or if the labeling intensity did not fall clearly into one category. Images were captured using an Olympus BX45 light microscope (Olympus Corp, Japan) and CellSens software (Olympus).

## 3. Results

For tissue sections processed after 8 hours of fixation for all glyoxal fixatives and NBF, results from H&E, special stains, and IHC were comparable. Marked differences were noted for some staining results, however, in tissue sections which underwent glyoxal fixation for 1 month and 4 months. It must be noted that all 4 glyoxal fixatives, although produced by different manufacturers and containing one or more undisclosed added reagents, performed similarly to each other. One of the most marked changes in tissues fixed with glyoxal for 1 or 4 months was the presence of pigment formation in liver and splenic

tissues (Figure 1). Skin, tongue, small intestine, breast, and lung samples exhibited little to no pigment formation. The pigment was easily removed by treating sections in an alkaline alcohol solution prior to staining, but frequently resulted in tissue loss from slides during subsequent staining.

The following special stains demonstrated no differences among fixatives used or length of fixation: melanin bleaching using either 10% hydrogen peroxide or potassium permanganate followed by oxalic acid, Verhoeff van Gieson, reticulin, Fontana Masson, mucicarmine, colloidal iron, and alcian blue. For H&E staining, all fixatives permitted excellent differential staining for specimens fixed for 8 hours. However, for 1-month and 4-month glyoxal-fixed specimens, hematoxylin uptake was markedly increased while eosin cytoplasmic labeling was almost non-existent (Figure 1). For the trichrome stain, the glyoxal fixatives demonstrated more vibrant red and blue as compared to NBF at 8 hours fixation. All glyoxal tissues displayed similar coloration to NBF at 1 month fixation but exhibited reduced color intensity as compared to NBF at 4 months fixation. For the Wright stain samples fixed at 8 hours, all clearly demonstrated mast cells. While the mast cells continued to stain well in glyoxal-fixed samples at 1 month and 4 months, the background stain produced increased intensity thereby hindering mast cell identification. Although the reticulin stain labeled collagen type III fibers appropriately under all fixative conditions, the tissues fixed in glyoxal solutions were cleaner appearing due to lack of intact red blood cells which in NBF tissues took up the nuclear fast red stain. One finding for the Fontana Masson stain was that for the 1- and 4-month glyoxal fixed tissues, the time needed to develop argentaffin reactions was reduced as compared to that of formalin-fixed tissues.

For IHC evaluations, long-term storage glyoxal results were mixed. All antibodies chosen to investigate produced maximum intensity staining (graded as 3) at 8 hours fixation for all fixatives. For some antigenic sites, antibody labeling was consistently reliable for all fixatives and fixation times (Table 21). However, due to the increased affinity for long-term glyoxal-fixed tissues for the IHC hematoxylin counterstain, contrast between positive labeling and counterstain was reduced in some instances. For other antigenic sites, long-term fixation demonstrated a marked reduction in labeling (Table 21) (Figure 2). Overall, the glyoxal fixatives performed similarly among IHC stains. Therefore, results were consolidated, and accessibility of antigenic sites was compared between glyoxal fixation and formalin fixation in general (Figure 3). Both formalin and glyoxal-fixed tissues exhibited decreased IHC labeling over time, but the reduction was more pronounced in the glyoxal-fixed tissues. All IHC evaluations were scored as 3 at 8 hours fixation. After 1 month fixation, the formalin-fixed tissues were still showing an intensity of 3 while glyoxal tissues averaged 2.4. At 4 months fixation, labeling of formalin-fixed tissues dropped to only 2.8 while glyoxal-fixed tissues dropped to 2.1 (Figure 3). For the 18 antibodies tested in this study, 7 (39%) maintained optimal labeling in glyoxal-fixed tissues for the 4 months duration of fixation. Another 7 (39%) antibodies demonstrated minimal loss of antigenicity, but continued to be deemed acceptable, while only 4 (22%) exhibited unacceptable antigenic labeling after 4 months fixation.

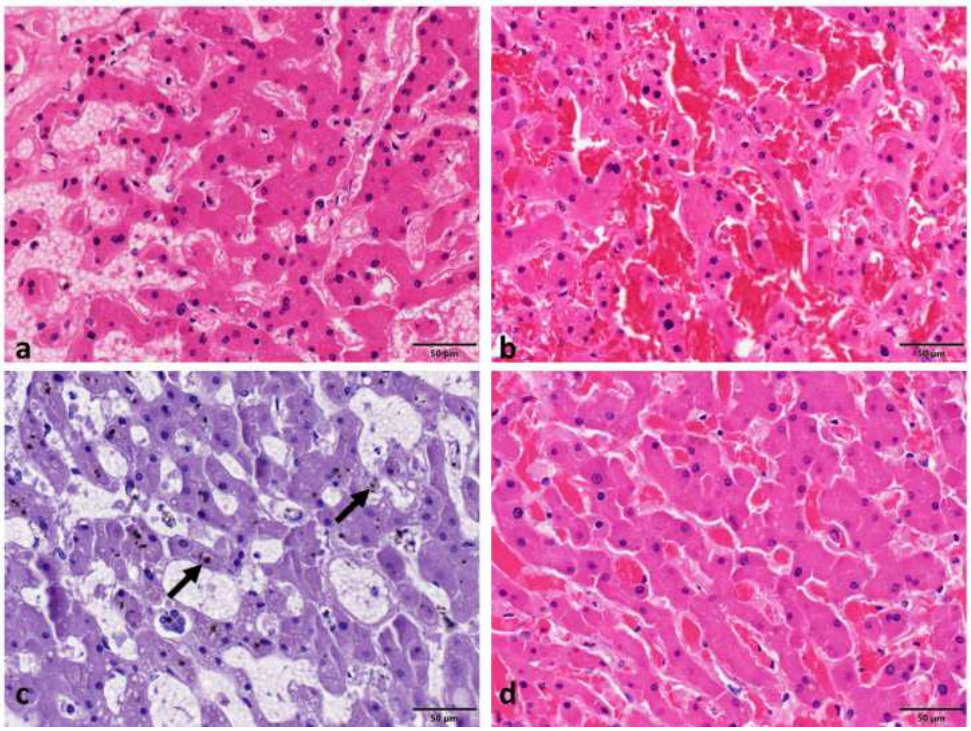


***Figure 1.*** *Liver sections stained with H&E. Sections fixed for 8 hours with (a) glyoxal fixative and (b) NBF. Sections fixed for 4 months in (c) glyoxal and (d) NBF. Only NBF-fixed tissues continued to demonstrate acceptable eosin intensity. Sections fixed with glyoxal fixatives for 1 month or 4 months exhibited strong basophilic cytoplasmic stain. Additionally, pronounced pigment deposits were seen in all glyoxal-fixed liver and spleen sections (arrows). Scale bar = 50 µm. NBF = Neutral buffered formalin.*

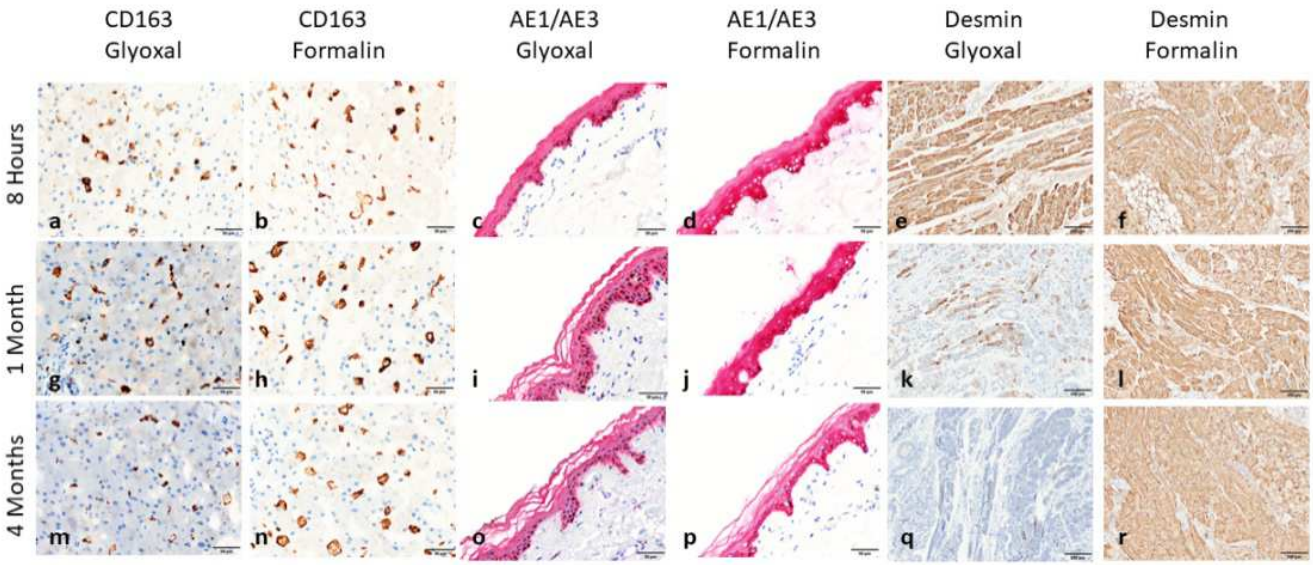


***Figure 2.*** *Immunohistochemistry assays for (a, b, g, h, m, n) anti-CD163, (c, d, i, j, o, p) anti-AE1/AE3, and (e, f, k, l, q, r) anti-desmin undergoing fixation for (a, b, c, d, e, f) 8 hours, (g, h, i, j, k, l) 1 month, (m, n, o, p, q, r) and 4 months. Glyoxal fixed specimens demonstrated negligible loss of antigenicity for CD163 (g, m), negligible loss of antigenicity for AE1/AE3 (i, o), but marked loss of antigenicity for desmin (k, q). Note hematoxylin counterstain in glyoxal tissues fixed 1 month and 4 months (g, i, k, m, o, q) is more intense than in sections fixed in glyoxal 8 hours only (a, c, e) or those fixed with formalin (b, d, f, h, j, l, n, p, r). Scale bar = 50 µm (a-d, g-j, m-p). Scale bar = 200 µm (e, f, k, l, q, r).*

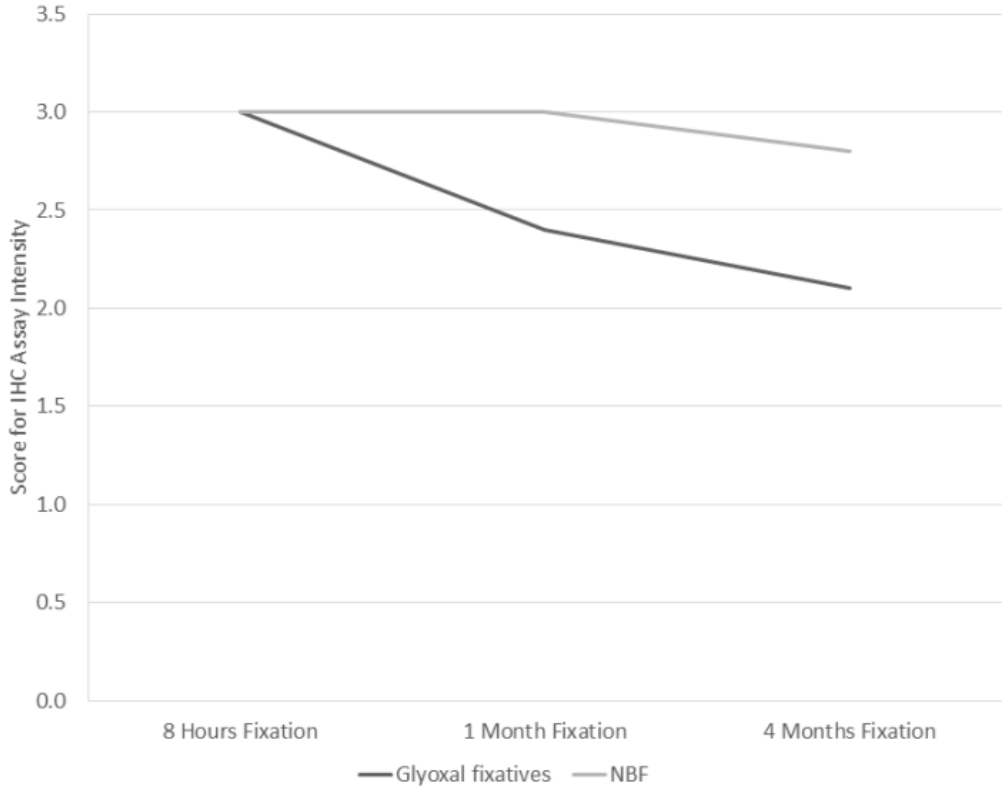


***Figure 3.*** *Average immunohistochemistry score for specimens fixed with glyoxal solutions and formalin. While formalin-fixed specimens scored the maximum score of 3 for all antibodies in tissues fixed for 8 hours or 1 month, there was a modest drop in antigenicity for specimens which underwent fixation for 4 months. Glyoxal-fixed specimens, however, exhibited a more rapid decline in antigenic labeling.*

## 4. Discussion

Special stain methods and routine H&E stain have been found to work well after long-term formalin fixation [6, 9]. Likewise, ultrastructural studies with electron microscopy have been found acceptable after long-term fixation with NBF or formalin-gluteraldehyde buffered solutions [2, 10]. For molecular testing in tissues undergoing long-term fixation, viral and human DNA extraction was found to be

strongly reduced as compared to samples which were unfixed or underwent short-term fixation only [4, 9, 12].

In glyoxal-fixed tissues processed immediately, red blood cells are lysed and eosinophilic granules in granulocytes, Paneth cells, and pancreatic exocrine cells are no longer identified easily by bright eosin stain, in contrast to tissues fixed in formalin. It has also been suggested that due to the low pH necessary for maintaining fixative properties of glyoxal, iron may be dissolved from bone marrow and liver tissues [5] and that breast calcifications necessary to verify correct biopsy sampling in breast cases may be diminished or removed [19]. Outside of these few negative aspects, no differences are seen with H&E stain between formalin-fixed tissues and glyoxal-fixed tissues when subjected to solution for 8 hours. However, tissues remaining in glyoxal for more than 2 weeks take on a strongly basophilic stain which does not correct with decreased time in hematoxylin or increased time in eosin [5]. The current study substantiates several of these findings including the overall intense basophilia of cell cytoplasm seen in tissues fixed for ≥1 month. Attempts to rectify the lack of nuclear/cytoplasmic contrast were made initially by decreasing staining time in hematoxylin while increasing time in eosin (Table 13, Figure 4b), similar to Dapson [5], and with correspondingly unsatisfactory results. Thereafter, a large variety of red dyes frequently used in the routine special stains laboratory were trialed in place of eosin with the most successful being basic fuchsine (Table 14, Figure 4c), picrosirius red (Table 15, Figure 4e), and Safranine O (Table 16, Figure 4f). Particularly the 0.13% basic fuchsine stain and 0.06% safranine O, both basic dyes, produced very vibrant cytoplasmic staining necessitating reduction in stain concentration and staining time.

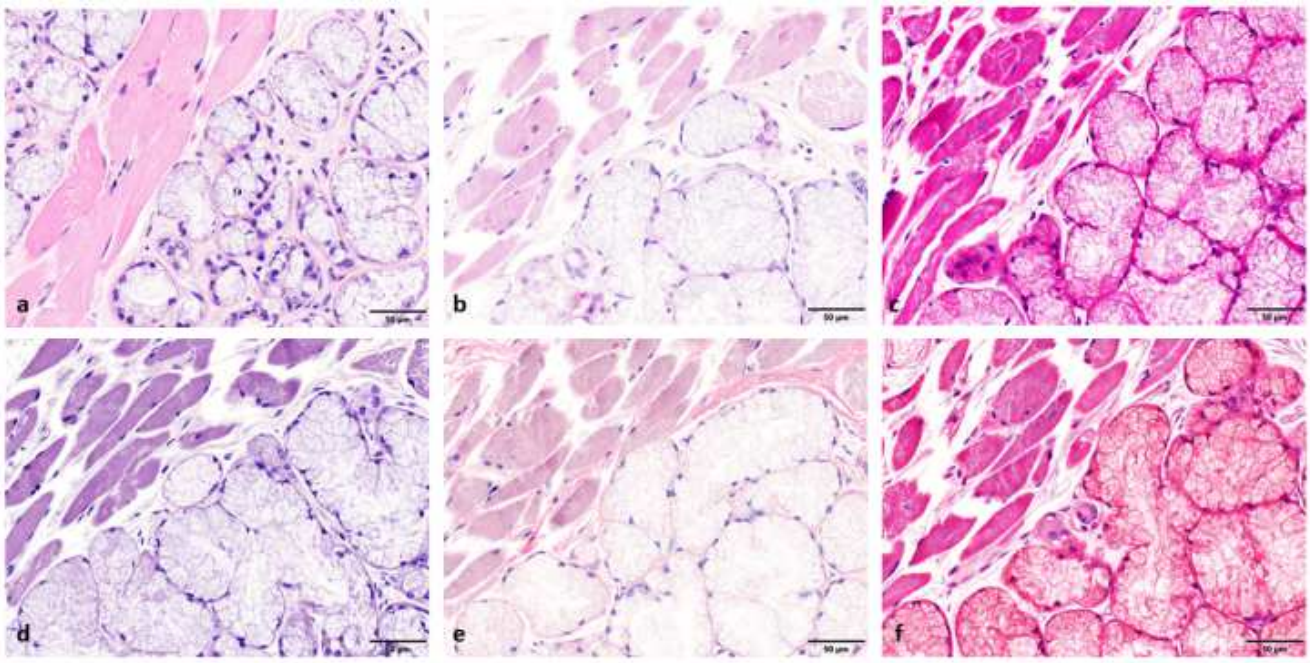


***Figure 4.*** *Tongue stained with H&E (a, b) and modified H&E stains (b, c, e, f). Specimens fixed 4 months in formalin (a) continued to appear well-stained while specimens fixed for 4 months in glyoxal (d) exhibited marked cytoplasmic basophilia. Modifications to improve eosinophilia of cytoplasm in tissues fixed in glyoxal for 4 months included decreasing hematoxylin stain time while increasing eosin stain time (b), substituting basic fuchsine for eosin (c), substituting picrosirius solution for eosin (e), and substituting Safranine O for eosin (f). Scale bar = 50 µm.*

Fixation in acidic formalin solutions is known to produce black acid hematin pigment which can be removed by treating tissues sections in an alkaline alcohol solution prior to staining [23]. While the glyoxal pigment found in tissues fixed ≥1 month was marked in liver and spleen sections, it was negligible or absent in skin, tongue, small intestine, skeletal muscle, breast, and lung. Additionally, the pigment could be removed by treating the sections with alkaline alcohol for 10 minutes after deparaffinization but prior to staining, similar to the process used to remove formalin pigment.

Special stain techniques, with a few exceptions, demonstrated no deleterious effects of long-term storage in glyoxal or formalin. Melanin bleaching using either 10% hydrogen peroxide or potassium permanganate followed by oxalic acid, Verhoeff van Gieson, reticulin, Fontana Masson, mucicarmine, colloidal iron, and alcian blue all demonstrated excellent staining after 4-months fixation. The glyoxal-fixed tissues demonstrated clearer reticulin fiber stain due to the lysing of the red blood cells in the liver sinusoids, and the Fontana Masson stain required shorter incubation time (10 minutes for glyoxal-fixed vs. 30 minutes for formalin-fixed tissues) for appropriate staining. The modified trichrome stain produced inferior results in glyoxal tissues fixed ≥1 month by exhibiting paler cytoplasm and collagen. However, no attempts were made to modify the protocol to achieve improved staining, an endeavor which would likely prove worthwhile. The Wright stain, also, displayed inferior results in tissues which underwent glyoxal fixation ≥1 month. However, the problem was not a lack of mast cell staining, but increased uptake of counterstain by the background tissue

elements which provided poor contrast to the mast cells.

Several previous studies have demonstrated a range of deleterious effects on IHC in tissues stored in fixative for extended periods of time. Although bone marrow biopsies fixed in buffered methanol-formalin solution for up to 12 days exhibited consistent IHC results [1], it may be successfully argued that fixation of 12 days is not by current standards considered to be a long-term fixation period, as tissues must be retained a minimum of 14 days per CAP accreditation standards. For bone specimens obtained during space flight and stored in 10% neutral buffered formalin for periods ranging from 4 hours to 1 year and then processed to methyl methacrylate, IHC and safranine O results were acceptable after 1 month, exhibited reduced labeling at 3 months, and demonstrated unacceptable labeling at 6 months [8]. For breast carcinomas stored in formalin for 7-154 days, investigators found that c-erb-b2 lost signal intensity after 20 days storage, but that most other markers used in breast cases did not lose intensity until approximately 42 days had passed [6]. Mastracci et al. determined that IHC labeling decreased in chromogenic intensity at 1 month storage for all antibodies requiring heat pretreatment [9]. However, antibodies requiring no pretreatment or enzymatic pretreatment continued to label appropriately at the 12-month interval. IHC stains which did not undergo heat-induced antigen retrieval steps demonstrated marked loss of antigenicity in samples fixed for ≥3 days [3]. While most studies describing effects of long-term fixation refer to formalin fixation primarily, one study did describe the effects of long-term glyoxal fixation. Fluorochrome labeling in bone specimens stored for 3 months in a glyoxal fixative continued to show appropriate labeling. However, the same study showed that whereas specimens fixed in formalin were acceptable at 1 year, those fixed in glyoxal were not [7]. For IHC results, the current study found that antibody labeling at 4 months fixation rivaled that of formalin fixation 78% of the time. Of the 18 antibodies tested, however, 4 (22%) were no longer reliable at 4 months fixation with glyoxal, but all continued to show adequate labeling in tissues fixed for 4 months in formalin. It has been suggested that diminished antigenic labeling in specimens fixed long-term in formaldehyde may be due to excessive cross-linking of tissue molecules that becomes partially irreversible. Because the mechanism of glyoxal fixation differs slightly with fewer cross-links formed, it may be the acidic pH of the glyoxal solutions which degrades tissue elements over time, destroying antigenic sites. When observing the keratinized stratified squamous epithelium of the skin labeled with AE1/AE3 antibody, and also in 4-month formalin-fixed skin, it is noted that the upper epithelial layers of the stratum corneum have become separated, possibly due to protein degradation.

## 5. Conclusion

Special stains including 2 melanin bleaching protocols followed by H&E, modified trichrome, Verhoeff van Gieson, mucicarmine, alcian blue, colloidal iron, reticulin, Wright, and Fontana Masson stains performed on tissues fixed in glyoxal for 8 hours to 4 months exhibited comparable results with minor differences only. The increased basophilia and decreased eosin uptake for the H&E stain in tissues fixed 1-4 months in glyoxal-based solutions were rectified by substituting Safranin O or basic fuchsine for eosin. For the IHC antibodies assayed, all worked optimally in formalin-fixed and glyoxal-fixed tissues after 8 hours of fixation, but appropriate labeling decreased marginally in formalin-fixed tissues at 4 months and the decrease was slightly more pronounced in glyoxal-fixed tissues. Taken together, the results of the current study suggest that tissues fixed up to 4 months in glyoxal solutions demonstrate, with a few exceptions, reliable special staining and IHC results.

## Funding Sources

None

## Conflicts of Interest

The authors declare that they have no competing interests.

## Study Approval

This study was approved by the University of Tennessee Health Science Center Internal Review Board IRB number 21-08027-NHSR.

## Acknowledgements

We extend our very sincere appreciation to the exceptional institutions, Doctors Anatomic Pathology Services, St. Bernards Healthcare, Methodist Le Bonheur Healthcare, St. Jude Children's Research Hospital, University of Tennessee DermPath, and Idexx laboratories, for their continued support of our student education and research efforts.

## References

[1] Werner M, Kaloutsi V, Buhr T, Bernhards J, Georgii A. 1992. Immunohistochemical examination of routinely processed bone marrow biopsies. Pathol. Res. Pract. 188 (6): 707-713.

[2] Collins B, MacNichol E. 1978. Long term fixation for histological studies. Microsco Acta. 81 (2): 155-158.

[3] Leong A, Gilham P. 1989. The effects of progressive formaldehyde fixation on the preservation of tissue antigens. Pathology. 21: 266-268.

[4] Kim O. 2003. Effect of fixation time and freeze-thaw cycles on the molecular analysis of viral DNA. J. Vet. Sci. 4 (2): 203-204.

[5] Dapson RW, Feldman AT, Wolfe D. 2006. Glyoxal Fixation and Its Relationship to Immunohistochemistry. J Histotechnol. 29 (2): 65-76.

[6] Arber D. 2002. Effect of prolonged formalin fixation on the immunohistochemical reactivity of breast markers. Appl Immunohistochem. Mol. 10 (2): 183-186.

[7] Clark N. 2002. Fluorochrome Labeled Bone (Part I): Long-term Fixation Results, J. Histotechnol. 25 (2): 101-103.

[8] Merrell G, Troiano N, Coady C, Kacena M. 2009. Effects of long-term fixation on histological quality of undecalcified murine bones embedded in methylmethacrylate. Biotech Histochem. 80: 3-4, 139-146.

[9] Mastracci L, Gambella A, Bragoni A, Pigozzi S, Pastorino L, Vanni I, Tosi I, Campora M, Fiocca R, Grillo F. 2019. Coping with formalin banning in pathology: under vacuum long-term tissue storage with no added formalin. Histochem. Cell Biol. 151: 501-511.

[10] Liu X, Schumann C. 2014. Optimization of electron microscopy for human brains with long-term fixation and fixed-frozen sections. Acta Neuropathol. Commun. 2: 42.

[11] Pikkarainen M, Martikainen P, Alafuzoff I. 2010. The effect of prolonged fixation time on immunohistochemical staining of common neurodegenerative disease markers. J Neurophathol Exp Neural. 69 (1): 40-52.

[12] Nagai M, Minegishi K, Komada M, Tsuchiya M, Kameda T, Yamada S. 2016. Extraction of DNA from human embryos after long-term preservation in formalin and Bouin's solutions. Congenit. Anom. 56: 112-118.

[13] Hopwood D. 1969. A comparison of the crosslinking abilities of glutaraldehyde, formaldehyde and α-hydroxyadipaldehyde with bovine serum albumin and casein. Histochemie. 17: 151-161.

[14] Dapson R. 2007. Glyoxal fixation: how it works and why it only occasionally needs antigen retrieval. Biotech Histochem. 82 (3): 161-166.

[15] Criswell S, Altman S, Peeler C, Drake T, Lazar C, Douglas S, DeJarnatt V. 2021. Glyoxal fixation: an immunohistochemistry assay evaluation. J Histotechnol, DOI: 10.1080/01478885.2021.1996125.

[16] Lassalle S. Hofman V, Ilie M, Gavric-Tanga V, Breast P, Havet K, Butori C, Selva E, Santini J, Mograbi B, et al. 2009. Assessment of morphology, antigenicity, and nucleic acid integrity for diagnostic thyroid pathology using formalin substitute fixatives. Thyroid. 19 (11): 1239-1248.

[17] Gatta L, Cadei M, Balzarini P, Castriciano S, Paroni R, Verzeletti A, Cortellini V, De Ferrari F, Grigolato P. 2012. Application of alternative fixatives to formalin in diagnostic pathology. Eur J Histochem. 56: e12.

[18] DeJarnatt V, Criswell S. 2020. Glyoxal: a proposed substitute for formalin in H&E and special stains, J. Histotechnology, 44 (1): 37-45.

[19] Umlas J, Tulecke M. 2004. The effects of glyoxal fixation on the histological evaluation of breast specimens. Hum Pathol. 35 (9): 1058-1062.

[20] Paavilainen L, Edvinsson A, Asplund A, Hober S, Kampf C, Ponten F, Wester K. 2010. The impact of tissue fixatives on morphology and antibody-based protein profiling in tissues and cells. J Histochem Cytochem. 58 (3): 237-246.

[21] Richter K, Revelo N, Seitz K, Helm M, Sarkar D, Saleeb R, D'Este E, Eberly J, Wagner E, Vogl C, et al. 2017. Glyoxal as an alternative fixative to formaldehyde in immunostaining and super-resolution microscopy. The EMBO J. 37: e95709.

[22] Celikkan F, Mungan C, Sucu M, Uysal F, Hayme S, Hayme S, Kuscu N, Ozkavukcu S, Celik-Ozenci C, Can A. 2020. PFA is superior to glyoxal in preserving oocyte, embryo, and stem cell proteins evidenced by super-resolution microscopical surveys of epitopes. J Assist Reprod Gen. 37: 369-384.

[23] Cook H. Manual of histological demonstration techniques. London: Butterworths; 1974.