FIXATION AND HISTOLOGICAL TECHNIQUES FOR THE RAT MODEL OF ALZHEIMER'S DISEASE

by

Reagan Jones

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FIXATION AND HISTOLOGICAL TECHNIQUES

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ALZHEIMER'S DISEASE

Project Approved:

Supervising Professor: Michael Chumley, Ph.D.

Department of Biology

Supervising Professor: Gary Boehm, Ph.D.

Department of Psychology

Matt Hale, Ph.D.

Department of Biology

Anna Petursdottir, Ph.D.

Department of Psychology

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ABSTRACT

Alzheimer's disease (AD) is a progressive form of dementia characterized by severe memory loss and protein anomalies in the brain, the severity of which is strongly correlated with a variety of genetic and environmental factors currently being studied. Animal models of AD allow for precise investigation of pathological mechanisms involved in the disease process, which provides valuable insight into potential etiology, treatments, and early indicators of the disease. This project focuses specifically on the Formalin-Fixed Paraffin-Embedded (FFPE) tissue processing method for the analysis of Alzheimer's disease-like pathology in the brains of Tg-F344 rats. These animal subjects express mutant human amyloid precursor protein (APPsw) and presenilin 1 (PS1ΔE9) genes, both of which have been found to drive amyloid beta accumulation and cause early-onset familial AD in humans. Modification of the reagents and techniques used for embedding, sectioning, and staining tissue in this method can greatly affect the integrity and morphology of the samples being studied. The lack of a standardized, universal FFPE protocol due to the varying nature of sample tissues and instruments requires that techniques be adapted to suit the needs of individual laboratories. The goal of the current study is to identify an efficient and effective method to obtain well-preserved rat brain tissue samples that are readily available for histochemistry and analysis in our laboratory, as this is essential to understanding the underlying pathology of AD and how it correlates to genetic and environmental factors.

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder of aging characterized by gradual memory loss and cognitive decline. According to 2021 statistics published by the Alzheimer's Association, AD is the fifth-leading cause of death among Americans aged 65 and older, and an estimated 6.2 million within this demographic are living with the disease today. The two primary pathological indicators of AD are the formation of amyloid beta plaques and neurofibrillary tau tangles, which aggregate in neuronal cells and disrupt cognitive function. Current research using animal models of AD focuses on how various environmental and genetic factors influence pathology and disease progression in the brain, with the hopes of better understanding the underlying causes and early indicators of the disease and finding potential therapeutic targets (1).

Evaluation of AD-like pathology in animal models allows researchers to comparatively analyze the severity of the disease between experimental and control groups. Several *ex vivo* and *in vitro* methods exist to analyze amyloidosis in brain tissue, including Immunohistochemistry (IHC), which uses antibodies to bind amyloid fibrils; fluorescent staining of amyloid aggregates with Thioflavin T/S; circular dichroism to determine the protein secondary structure in amyloid aggregates; Fourier transform IR spectroscopy; nuclear magnetic resonance; X-ray diffraction; cryoelectron microscopy; transmission electron microscopy; and atomic force microscopy (2). The favored procedure for histological confirmation of amyloid plaques is the Congo Red (CR) stain visualized using polarized light microscopy or by red coloration in conventional bright field microscopy (3). The thin tissue sections required for the visualization of brain ultrastructure can be obtained through several techniques, including agarose-embedded vibrating microtome

(vibratome) sectioning, plastic resin-embedded ultra-thin sectioning, formalin-fixed cryosectioning, and Formalin-Fixed Paraffin-Embedded (FFPE) microtome sectioning.

A vibratome is an instrument that uses a vibrating razor blade to section tissue into thin slices for histochemical analysis. It is advantageous over a microtome because fixed microtome blades often compress the dehydrated tissue sections during cutting, which disrupts the structure of the sample. Agarose is the most common tissue embedding medium chosen for vibratome sectioning, and it helps keep the tissue soft and stable during cutting to prevent compression. Another appeal to the vibratome method is that tissue does not need to be dehydrated prior to embedding, which helps preserve the original tissue structure. However, collecting vibratome sectioned tissue is much more tedious and time-consuming compared to other methods since only delicate single sections are cut at one time. Sections are also thicker than those obtained through the paraffin-embedded microtome method, which makes IHC reagent penetration difficult (4). Another disadvantage is bulky storage requirements. Vibratome sections can be stored unstained at 4°C in a buffer but must be kept from dehydrating. Alternatively, the sections can be stored in a freezing medium that prevents ice crystal formation while maintaining temperatures as low as -20°C. This preserves the tissue for much longer and prevents pathogens and fungi from growing in the liquid media, but storage in this manner is bulky and expensive (5).

Another method involving the ultrathin sectioning of plastic embedded tissue samples at a thickness between 20-150 nm is the favored method for transmission electron microscopy. The low-voltage electron microscopes require that ultrathin sections be cut from fixed, dehydrated tissue samples embedded in a plastic resin using glass or diamond knives to achieve such high-resolution visualization of microscopic cell structures (6). Like paraffin-embedded specimens,

resin-embedded ultrathin sections can be stored at ambient temperature for several years. The downside to this method is the amount of time required to obtain suitable sections and the difficulty in manipulating the delicate resin-embedded samples, which are more prone to alteration compared to other methods (7).

Frozen tissue sections, often fixed in formalin similarly to paraffin-embedded tissue, can be sectioned using a cryostat, an instrument that contains a tissue microtome (cryotome) within a cooling chamber that allows the process of cutting to occur at low temperatures between -10° to -20° C (8). Although cryosections are physically less stable than paraffin or resin-embedded sections due to the need for storage at freezing temperatures, they have a much higher degree of molecular preservation thanks to the relative lack of interfering reagents (9). Students in Dr. Mauricio Papini's lab studying the comparative neuroscience of emotion and learning at TCU have contributed a great deal of effort in refining the process of cryosectioning for rat brains with excellent results in downstream histochemical and immunohistochemical analysis.

This project focuses specifically on refining the Formalin-Fixed Paraffin-Embedded (FFPE) method as an alternative sectioning technique for use in TCU laboratories studying rat brains. Using the FFPE method, harvested post-mortem tissue is perfused, dissected, then preserved in a fixative, and finally embedded in a paraffin wax block. The block containing the intact tissue sample can then be sectioned and stained for specific proteins or structures of interest (10). This study utilizes the brains of Tg-F344 rats expressing mutant human amyloid precursor protein (APPsw) and presenilin 1 (PS1ΔE9) genes, which have both been found to drive amyloid beta accumulation and cause early-onset familial AD (11). Brains are processed using the above method, sectioned using a rotary microtome, and stained for amyloid beta plaques.

The FFPE method has several advantages in that it allows for both morphological and molecular tissue analysis, stabilizes tissues for long periods of time at room temperature, and preserves the integrity of the original specimen remarkably well (12). AD pathology can be observed at both a macro and microbiological level of brain tissue. Structural tissue alterations, including cortical atrophy, enlarged ventricles, and cerebrovascular disease often accompany common microscopic features of AD, such as neurofibrillary tangles, amyloid plaques, cerebral amyloid angiopathy, granulovacuolar degeneration, Hirano bodies, glial response, neuronal loss, and synapse loss—all of which are preserved in FFPE tissue sections (13). An additional benefit is the ability to isolate sequential thin sections from the same tissue sample such that two sequential sections likely will contain most of the same cells and will be within the same brain regions, allowing for different targets to be stained on similar sections. This leads to the generation of more data for the same cells and brain regions. FPPE also provides several logistical advantages over vibratome sections and fresh frozen tissue samples used in cryosectioning in that specimens can be stored at room temperatures for decades on end without the need for specialized storage equipment, since the formaldehyde used during fixation crosslinks proteins in the tissue, essentially locking them into place (14).

Despite the numerous advantages of the FFPE tissue processing method, histology of brain tissue is not without difficulty. Due to the variable nature of tissue samples from different organisms, no single universal protocol exists for FFPE tissue processing. Even within widely accepted protocols for certain tissue types, many factors can alter the outcome of the tissue sample. The density of the tissue, volume of fixative used, and time spent in the fixative can all dramatically affect the resultant specimen and subsequent proteomic analysis. Both over-fixation and under-fixation can degrade the specimen and alter histomorphology and

immunohistochemistry. The process of embedding the tissue in paraffin similarly introduces hurdles in the recovery of biomolecules, although to a lesser extent than the process of fixation. Quality of reagents, time spent in sequential dehydration steps, and temperature throughout all affect sample integrity (15). Many studies have reported that histological processing of FFPE tissue sections can lead to masking or denaturing of epitopes that antibodies used in IHC bind to, and antigen retrieval methods often require enzymatic digestion or heat which can irreparably damage the tissue (16). For CR staining, the concentration of CR solution used, time rinsed in water, and immersion in sequential ethanol stations affect the visualization of stained amyloid beta peptide against the background of nonspecific cellular structures (17). Given all these variables, each technique must be fine-tuned for specific laboratories and specimens to obtain high-quality, stained tissue sections for histopathological analysis.

The purpose of this study is to compare the effects of different FFPE and staining protocol modifications on the analysis of Alzheimer's plaque formations within the rodent brain to streamline the process for future laboratory use.

MATERIALS AND METHODS

TISSUE PREPARATION

This study utilized 7-month-old TgF-344 AD rats housed in the Texas Christian University vivarium in accordance with approved protocols of the Institutional Animal Care and Use Committee (IACUC). Rats were anesthetized with pentobarbital and euthanized via transcardial perfusion with phosphate-buffered saline (PBS). Separated brain hemispheres were fixed in 4% paraformaldehyde (PFA) for 14 days to halt tissue degeneration and preserve cellular structures.

Tissue specimens were processed using a Leica TP1020 automated tissue processor (Leica Microsystems, Bannockburn, IL) in which the tissue encased in a plastic cassette underwent dehydration, clearing, and wax infiltration by passing through separate wells containing different reagents for pre-programmed periods of time. Since paraffin is immiscible with water, the tissue was first dehydrated in an ascending series of ethanol concentrations (American MasterTech Scientific, Lodi, CA) to allow for complete infiltration of the paraffin wax. The ethanol must also be removed since it is largely immiscible with paraffin. In this study, an ethanol clearing agent known as Sub-X (Leica Microsystems, Bannockburn, IL), a less toxic substitute for Xylene, was used. The tissue was placed in the two subsequent Sub-X wells for one hour. After dehydration and clearing, histological paraffin can infiltrate the tissue. The specimen was placed in three paraffin stations for one hour each at a temperature of 60° C. The order and duration for each processing step are outlined in the procedure below:

1. 70% EtOH – 1 hour
2. 80% EtOH – 1 hour

3. 95% EtOH – 1 hour
4. 95% EtOH – 1 hour
5. 100% EtOH – 1 hour
6. 100% EtOH – 1 hour
7. Sub-X – 1 hour
8. Sub-X – 1 hour
9. Paraffin – 1 hour
10. Paraffin – 1 hour
11. Paraffin – 1 hour

PARAFFIN EMBEDDING

After thorough paraffin infiltration, the tissue was embedded in a paraffin wax block using a Leica HistoCore Arcadia paraffin embedding device (Leica Microsystems, Bannockburn, IL). The specimen was carefully oriented in a mold to determine the plane of section and topped with a plastic cassette. The mold was then filled with molten paraffin and allowed to cool into a solid block.

ROTARY MICROTOMY

A Leica RM2235 rotary microtome (Leica Microsystems, Bannockburn, IL) was used to cut the tissue into sagittal sections that were 8-10 microns thick. A ribbon of sections was then floated on the surface of a distilled water bath set at 42°C to allow for proper expansion of the tissue, which was often compressed by the knife blade during sectioning. Sections were then mounted on Gold Seal Ultrastick microscope slides (Ted Pella, Redding, CA), allowed to dry on a slide warmer for approximately 5 minutes, and stored at room temperature until staining was conducted.

STAINING

In this study, slides containing sectioned tissue were stained with Congo Red dye (Sigma-Aldrich, St. Louis, MO) to demonstrate amyloid aggregates. 500 mL of a saturated NaCl solution was added to 0.1g of Congo Red dye and the resulting Congo Red solution was stirred and filtered through a 0.45-micron membrane filter (18). Sections were deparaffinized, rehydrated, and stained using the reagents listed in the table below. Separate trials were run with adjusted durations in ethanol to promote better visualization of stained amyloid beta plaques:

Reagent	Trial 1	Trial 2
1. Sub-X	3 mins	3 mins
2. Sub-X	2 mins	2 mins
3. 100% Ethanol	1 min	1 min
4. 95% Ethanol	1 min	1 min
5. 80% Ethanol	1 min	1 min
6. Water	30 secs	30 secs
7. Alkaline Solution	20 mins	20 mins
8. Congo Red	30 mins	30 mins
9. 95% Ethanol	8 dips	4 dips
10. 100% Ethanol	8 dips	4 dips
11. Sub-X	1 min	1 min
12. Sub-X	2 mins	2 min
13. Sub-X	Hold	Hold

MICROSCOPY

Stained sections were first visualized at low magnification using a Zeiss Stemi305 (Carl Zeiss Microscopy, White Plains, NY) with integrated WiFi camera, and images were collected using the Zeiss Labscope software on an Apple iPad Pro. This allowed for basic visualization of brain tissue morphology. Subsequently, stained sections were visualized using a Zeiss AxioLab A.1 (Carl Zeiss Microscopy) outfitted with a Zeiss AxioCam ErC5s camera. Images were then color- and sharpness-enhanced using ImageJ software (19).

RESULTS

SECTIONING

Several troubleshooting steps were required with each new tissue sample sectioned (see **Figure 1**). Many minor adjustments, such as the blade clearance angle, section thickness, and the knife blade itself, were modified on the rotary microtome apparatus to achieve high-quality, intact sections suitable for histological staining.

Figure 1A depicts a connected ribbon of sectioned tissue that contains a pattern of visible knife lines pointed at by yellow arrows. This was a common issue that arose from a dull knife blade where paraffin buildup occurred and was easily fixed by changing the knife blade with each new tissue block that was sectioned, or, if necessary, multiple times during the sectioning of one tissue.

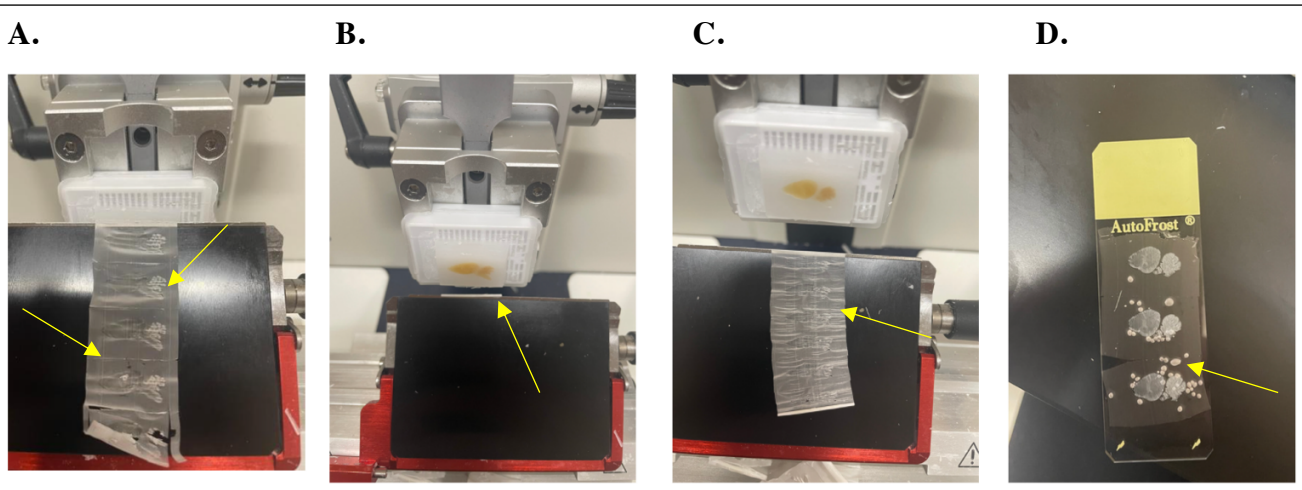

Figure 1. Common sectioning issues requiring troubleshooting. (**A**) Knife lines. (**B**) Curling. (**C**) Compression. (**D**) Bubbles

Figure 1B shows curling of the tissue as it is cut. This was a common problem encountered while attempting to section tissue thicker than 20 microns, but rarely an issue with a thickness under 10 microns. The majority of sections were cut between 8 and 10 microns for this reason and to allow better visualization of cellular structures using light microscopy.

Figure 1C depicts considerable compression of tissue during cutting. Some compression and wrinkling could be overcome by teasing sections with blunt forceps or floatation on a warm water bath set at 42° C: a temperature that is below the melting point of paraffin, but warm enough to allow the wax and the tissue within it to spread back to its original size. However, too much compression like that shown in **Figure 1C** led to permanent folds and wrinkles if not corrected properly. Common causes of this determined during the troubleshooting process were a dull knife blade, insufficient rehydration and cooling of the tissue block, and an incorrect clearance angle. As mentioned above, frequent changing of the knife blade resolved a lot of issues encountered. Additionally, rehydrating the tissue prior to and periodically during sectioning in an ice-water bath made the tissue less dry and prone to cracking, and reduced friction between the knife blade and the paraffin, leading to less compression and wrinkling. Another variable that needed adjustment was the knife tilt angle. If the angle was too great, there was more friction between the blade and the block of tissue because a greater surface area of the blade hit the wax block during cutting. We determined that a tilt angle of approximately 5 degrees consistently produced better quality sections.

Figure 1D shows bubbles trapped underneath tissue sections mounted on a microscope slide. Using charged microscope slides facilitated the adhesion of sections from the water bath onto the slide. Sections were retrieved slowly and carefully so that air bubbles were not introduced. Additionally, sections retrieved from the microtome with blunt forceps had to be very gently laid

on the water bath to prevent the trapping of air bubbles in this step between the tissue section and the surface of the water.

First attempts at microtomy were conducted on a coronal plane (see **Figure 2**). Minor wrinkling was observed since the section thickness was only 4 microns, but the tissue section was largely intact. Sectioning in the coronal plane consistently produced better quality and more intact tissue sections, although efforts were shifted to improve quality in the sagittal plane for several reasons. Firstly, sectioning in the sagittal plane allowed key brain regions such as the cortex, hippocampus, and cerebellum to be visualized simultaneously within one tissue section. Another reason was to better fit the needs of our laboratory and lessen the number of animal subjects used since one sagittal hemisphere was already needed for other assays conducted in our laboratory. Sagittal sections, however, are much harder to keep intact likely because of the larger cross-sectional size of the ventricles in this plane. As shown in **Figure 2** below, the coronal section appears much more morphologically intact than the sagittal sections, and only minimal cracking and tearing of tissue was observed (see **Figures 3A, 3B**).

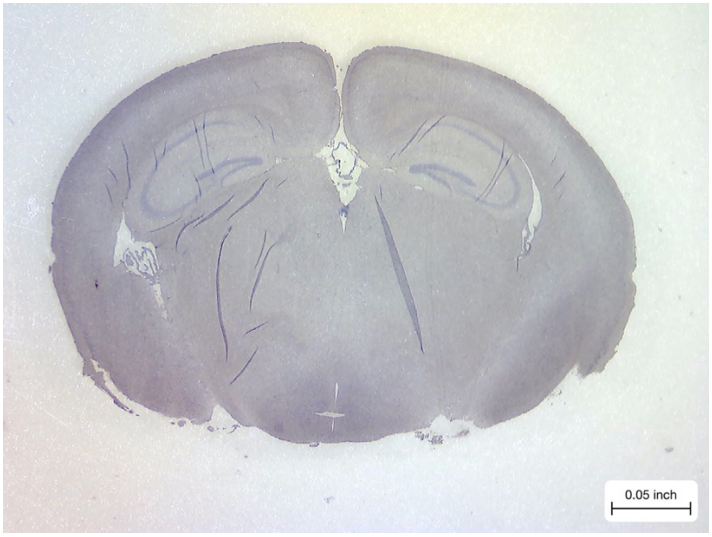

Figure 2. 20X magnification of a 4-micron coronal section of a TgF-344 rat cortex stained with both Hematoxylin and Congo Red.

A. **B.**

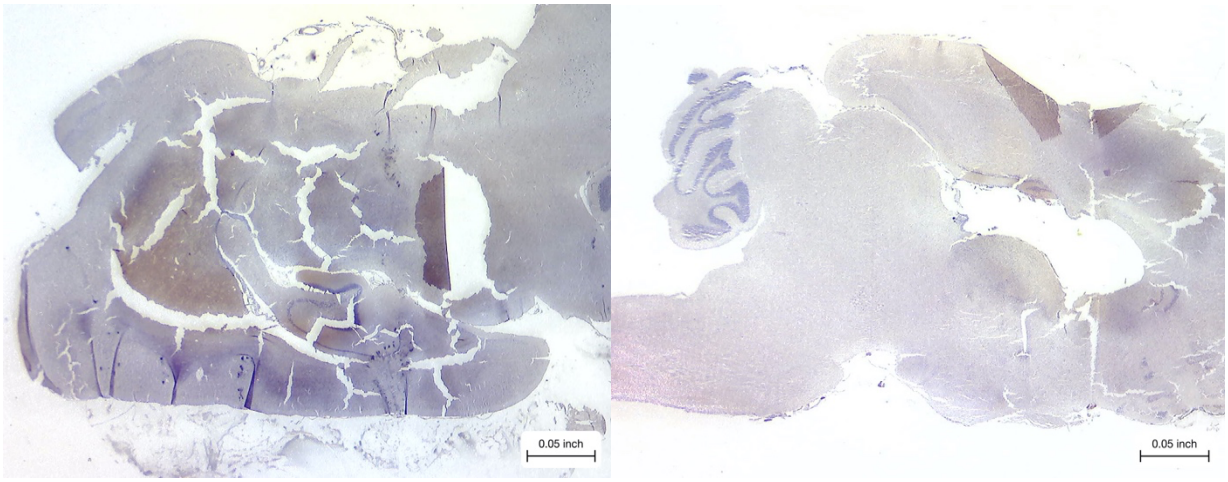

Figure 3. 20X magnification of sagittal sections of a TgF-344 rat brain stained with Hematoxylin and Congo Red. (**A**) 8-micron section obtained prior to sectioning troubleshooting. Followed Trial 1 of the staining procedure. (**B**) 8-micron section obtained after troubleshooting techniques were implemented and stained using the Trial 2 procedure.

After the implementation of each of the above troubleshooting steps, the quality of sagittal tissue sections greatly improved compared to initial attempts. **Figure 3A** depicts an early tissue section prior to microtomy troubleshooting, and **Figure 3B** shows great improvement in tissue quality (i.e., much less cracking and folding) after the above adjustments were made. There is still much room for improvement, given that coronal sections still appear more intact. More experience and diligent implementation of the above techniques will likely increase quality to an even greater extent in future microtomy attempts.

AMYLOID BETA PLAQUE VISUALIZATION

A. **B.**

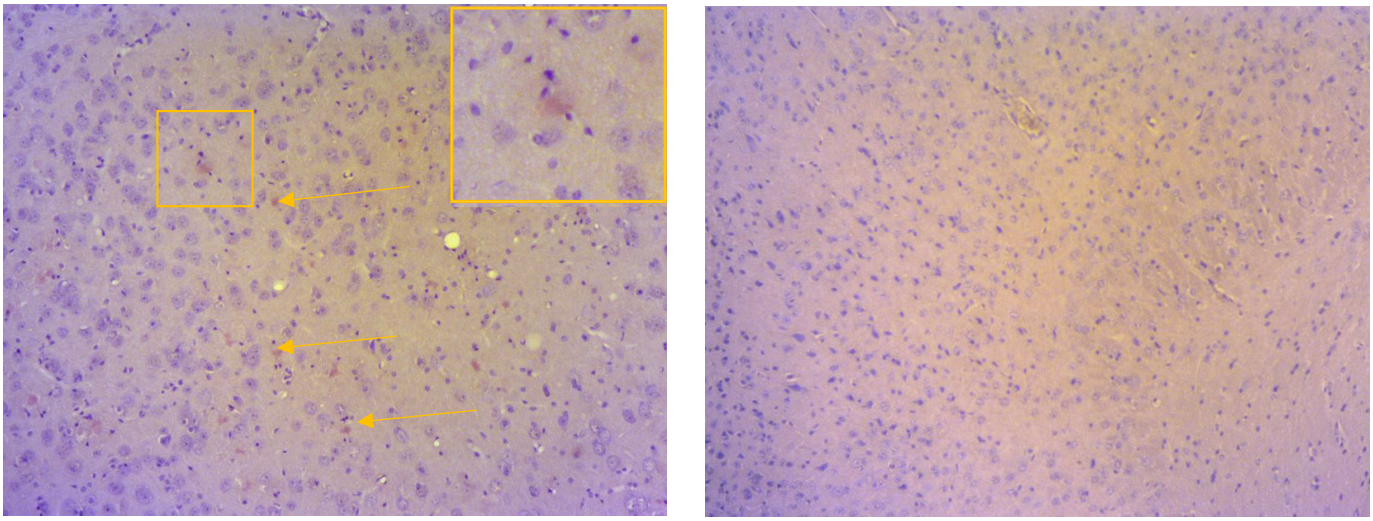

Figure 4. (A) 40X inset of a 5XFAD mouse cortex stained with Hematoxylin and Congo Red. Amyloid beta plaques are pointed to by yellow arrows. **(B)** 40X inset of a wild-type mouse cortex stained with Hematoxylin and Congo Red.

First attempts at staining were conducted successfully on 5XFAD and wild-type mice (**Figure 4**). Due to the need for rats as subjects in planned future studies for our laboratory, we wanted to try the same staining procedure on TgF-344 rats. However, Amyloid beta plaques could not be visualized in these subjects (see **Figure 5** below). First attempts at staining were conducted using the Trial 1 staining procedure outlined in the Methods section above, which was adapted from a 2006 publication in *Nature Protocols* (18). Amyloid beta aggregates could not be detected using light microscopy, and essentially all the Congo Red stain was washed off. We hypothesized, based on information in the *Nature* publication, that slides were rinsed too long in the final ethanol steps such that the stain was entirely stripped from the tissue (see steps 9 & 10 in the Trial 1 procedure). To try and correct this, another trial was run with only 4 dips in ethanol rather than 8 to decrease the total amount of time slides were rinsed with ethanol after immersion

in the Congo Red solution. The resulting slides from the second trial were much pinker, indicating that the Congo red dye successfully infiltrated the tissue and was not washed away by the ethanol, but plaques still could not be visualized under the microscope.

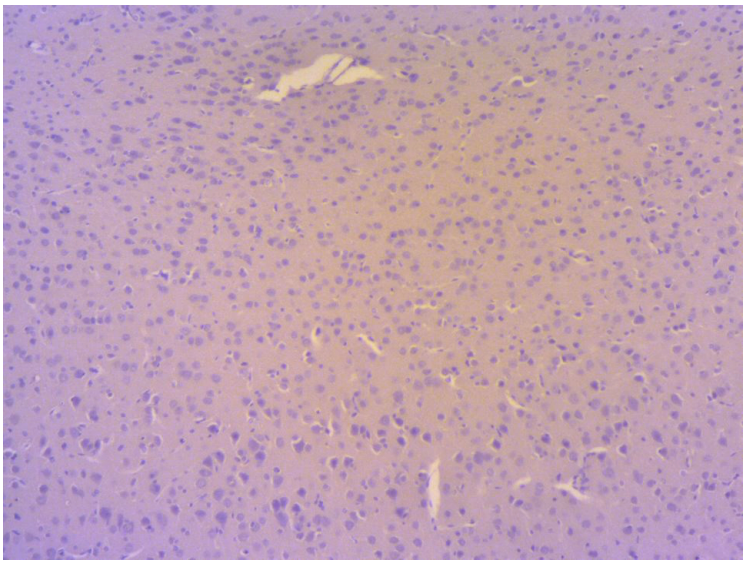

Figure 5. 40X inset of a TgF-344 rat cortex stained with Hematoxylin and Congo Red.

DISCUSSION

We have hypothesized several potential errors which would explain the lack of amyloid beta plaque visualization in transgene-positive rats. An easy explanation would be that the Congo Red powder dye used to make the staining solution is expired and no longer functional. Another explanation could be a procedural error at any point of fixation or tissue processing that prevents targeting of amyloid aggregates or destroys them altogether. Given that there are so many techniques and reagents used throughout the process, identifying the exact point of fault requires an enormous amount of trial and error. Tissues extracted from the rat brains may have been fixed too long in PFA prior to processing, which may have caused excessive cross-linking of proteins thereby preventing the binding of Congo Red to the amyloid beta plaques. Over-fixation may also lead to increased brittleness and dryness of tissues, which would explain the excessive cracking and wrinkling observed in tissue sections (20). Additionally, the dryness and brittleness of processed tissue could potentially be overcome by increasing the time of incubation in paraffin during the automated tissue processing step to allow for more thorough paraffin infiltration, which will enhance the ability to section tissues more smoothly. A final limitation in this study could be errors in animal genotyping, or that subjects used were too young, such that they had not yet developed amyloid pathology. To test this hypothesis, we hope to conduct fluorescent thioflavin staining (discussed briefly in the Introduction), with the remaining sections from the same subjects to definitively confirm the presence or absence of amyloid pathology in these subjects.

Overall, this study greatly improved microtomy techniques for paraffin-embedded tissue and identified future areas of improvement in the FFPE process, which will ultimately allow us to obtain high-quality tissue sections that can successfully be stained with Congo Red to target

and visualize amyloid beta plaques in the rat brain. Once successful, this method will serve as an invaluable tool to evaluate amyloid pathology in any future study involving animal subjects.

REFERENCES

(1) 2021 Alzheimer's disease facts and figures. (2021). *Alzheimer's & Dementia, 17*(3), 327-406. doi:10.1002/alz.12328

(2) Yakupova, E., Bobyleva, L., Vikhlyantsev, I., & Bobylev, A. (2019). Congo red and amyloids: History and relationship. *Bioscience Reports, 39*(1) doi:10.1042/BSR20181415

(3) Mollee, P., Renaut, P., Gottlieb, D., & Goodman, H. (2014). How to diagnose amyloidosis. *Internal Medicine Journal, 44*(1), 7-17. doi:10.1111/imj.12288

(4) Robertson N (2002) Vibratome sectioning of tissues: an alternative to paraffin methods. http://www4.ncsu.edu/~rgfranks/research/protocols/immuno%20histio%20chemistry/robertson%20vbratmimuno.Doc

(5) Shim K. (2011). Vibratome sectioning for enhanced preservation of the cytoarchitecture of the mammalian organ of Corti. *Journal of visualized experiments : JoVE*, (52), 2793. https://doi.org/10.3791/2793

(6) Nebesářová, J., Hozák, P., Frank, L., Štěpan, P., & Vancová, M. (2016). The cutting of ultrathin sections with the thickness less than 20 nm from biological specimens embedded in resin blocks. *Microscopy Research and Technique, 79*(6), 512-517. doi:10.1002/jemt.22659

(7) Verhertbruggen, Y., Walker, J. L., Guillon, F., & Scheller, H. V. (2017). A comparative study of sample preparation for staining and immunodetection of plant cell walls by light microscopy. *Frontiers in Plant Science, 8*, 1505. doi:10.3389/fpls.2017.01505

(8) Bullock, S., Balzer, B., & Marchevsky, A. (2015). Guide to the preparation of frozen sections. *Intraoperative consultation: Foundations in diagnostic pathology* (pp. 16-22). Philadelphia, PA: Saunders.

(9) The pros and cons of FFPE vs frozen tissue samples. Retrieved from https://www.geneticistinc.com/blog/the-pros-and-cons-of-ffpe-vs-frozen-tissue-samples

(10) Zhanmu, O., Yang, X., Gong, H., & Li, X. (2020). Paraffin-embedding for large volume bio-tissue. *Scientific Reports, 10*(1), 1-8. doi:10.1038/s41598-020-68876-5

(11) Cohen, R. M., Rezai-Zadeh, K., Weitz, T. M., Rentsendorj, A., Gate, D., Spivak, I., . . . Town, T. (2013). A transgenic alzheimer rat with plaques, tau pathology, behavioral impairment, oligomeric aβ, and frank neuronal loss. *The Journal of Neuroscience, 33*(15), 6245-6256. doi:10.1523/JNEUROSCI.3672-12.2013

(12) Matsuda, K. M., Chung, J., & Hewitt, S. M. (2010). Histo-proteomic profiling of formalin-fixed, paraffin-embedded tissue. *Expert Review of Proteomics, 7*(2), 227-237. doi:10.1586/epr.09.106

(13) Serrano-Pozo, A., Frosch, M. P., Masliah, E., & Hyman, B. T. (2011). Neuropathological alterations in alzheimer disease. *Cold Spring Harbor Perspectives in Medicine:, 1*(1) doi:10.1101/cshperspect.a006189

(14) Donczo, B., & Guttman, A. (2018). Biomedical analysis of formalin-fixed, paraffin-embedded tissue samples: The holy grail for molecular diagnostics. *Journal of Pharmaceutical and Biomedical Analysis, 155*, 125-134. doi:10.1016/j.jpba.2018.03.065

(15) Hewitt, S. M., Lewis, F. A., Cao, Y., Conrad, R. C., Cronin, M., Danenberg, K. D., . . . Warrington, J. A. (2008). Tissue handling and specimen preparation in surgical pathology: Issues concerning the recovery of nucleic acids from formalin-fixed, paraffin-embedded tissue. *Archives of Pathology & Laboratory Medicine, 132*(12), 1929-1935. doi:10.5858/132.12.1929

(16) Grillo, F., Pigozzi, S., Ceriolo, P., Calamaro, P., Fiocca, R., & Mastracci, L. (2015). Factors affecting immunoreactivity in long-term storage of formalin-fixed paraffin-embedded tissue sections. *Histochemistry and Cell Biology, 144*(1), 93-99. doi:10.1007/s00418-015-1316-4

(17) Rajamohamedsait, H. B., & Sigurdsson, E. M. (2012). Histological staining of amyloid and pre-amyloid peptides and proteins in mouse tissue. *Methods in Molecular Biology (Clifton, N.J.), 849* doi:10.1007/978-1-61779-551-0_28

(18) Wilcock, D. M., Gordon, M. N., & Morgan, D. (2006). Quantification of cerebral amyloid angiopathy and parenchymal amyloid plaques with congo red histochemical stain. *Nature Protocols, 1*(3), 1591-1595. doi:10.1038/nprot.2006.277

(19) Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018.

(20) Webster, J. D., Miller, M. A., DuSold, D., & Ramos-Vara, J. (2009). Effects of prolonged formalin fixation on diagnostic immunohistochemistry in domestic animals. *Journal of Histochemistry and Cytochemistry, 57*(8), 753-761. doi:10.1369/jhc.2009.953877