Multiplex immunofluorescence methods in neurodegenerative diseases

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Abstract

BACKGROUND: The ability to simultaneously localize multiple targets within human tissue sections would markedly facilitate the discovery and validation of molecular pathways underlying neurodegenerative diseases and the understanding of selective vulnerability. The main roadblock to this is successful elution of antibodies or other probes while maintaining tissue and antigen integrity. Although several multiplex techniques involving elution have been proposed, few have been applied or validated in the context of neurodegenerative diseases in human post-mortem tissue. Moreover, despite the increased complexity of these protocols, quality control steps for multiplex techniques have not been regularly or explicitly implemented in studies utilizing them.

NEW METHOD: Aiming to create protocols for multiplex localization of neurodegenerative processes without the need for specialized equipment, we evaluated several proposed techniques and created a pipeline tailored for the intricacies of human post-mortem brain tissue and suggest quality control steps to ameliorate concerns of cross-reactivity and false positives.

RESULTS: The proposed protocol using β-mercaptoethanol enables reliable primary and secondary antibody elution across multiple rounds of staining and minimizes the odds of cross reactivity while preserving tissue and antigen integrity.

COMPARISON WITH EXISTING METHODS: Our proposed method examines multiplex immunohistochemistry in the context of neurodegenerative proteinopathies, accounting for the intricacies of human post-mortem tissue. Namely, we account for the need to elute markers of highly aggregated proteins in tissue with significant autofluorescence, diminishing the possible signal-to-noise ratio. Additionally, it explicitly implements and suggests quality control steps to align with the increased complexity associated with multiplex histology.

CONCLUSIONS: Multiplex immunofluorescence techniques for studying neurodegenerative diseases in postmortem human tissue are feasible and can be implemented in laboratories lacking sophisticated equipment. Nevertheless, the evaluation of elution parameters in the optimization and validation phase of any experiment is prudent.

Keywords

Multiplex histology, Immunohistochemistry, Immunofluorescence, Neuropathology, Postmortem human brain tissue

1. Introduction

The biology underlying neurodegenerative diseases is intimately related to the complexities of the human brain. Deposition and aggregation of abnormally folded proteins is a common characteristic of neurodegenerative diseases, however animal models of this protein deposition do not feature the same cytoarchitectonics, stereotypical spread of pathology, or selective cellular vulnerability as human cases. Given the selective vulnerability underlying neurodegenerative diseases (Fu et al., 2018), this difficulty involved in developing models that recapitulate the full array of disease processes represents one of the biggest hurdles to elucidating fundamental biology and etiology. Even the best-probed molecular pathways of disease require the context of anatomy, human variation, disease stage, and even clinical syndrome to validate their relevance and contributions to disease etiology. Overcoming these hurdles can be aided by using human post-mortem tissue, thus allowing for direct observation of different proteinopathies and the classification of different cell-types. A variety of pipelines and methods have been developed to detect and quantify multiple targets in single tissue sections with each presenting various levels of associated costs, reliability, and flexibility.

For routine studies in molecular pathology or experimental neuropathology, a desired pipeline for post-mortem validation would be able to accommodate a variety of sample qualities and a variety of antibody combinations, use ubiquitous equipment and widely available reagents, be consistent and reliable, be realistically used in large sample sizes, and remain cost-effective. A proposed ideal pipeline for studying molecular pathology in neurodegenerative diseases would involve (1) identifying candidate molecular pathways; (2) validating antibodies or other probes to visualize the candidate pathways in readily accessible histological sections; (3) combining probes to inform on hypothesized interactions in a well-characterized clinico-pathological series; (4) efficient quantification and analysis of probes in the clinico-pathological series. Here we focus on step three of the pipeline, which has proven to be a major roadblock in traditional immunohistochemistry labs.

Traditional immunohistochemistry using chromogenic reporters limits investigators to studying two or three probes simultaneously (Dixon et al., 2015; Gown et al., 1986; Ilie et al., 2018; Lan et al., 1995; Nakane, 1968; Tramu et al., 1978). The advent of fluorescence microscopy expanded these capabilities by utilizing reporter molecules with more optic properties than chromogens such as 3,3’-Diaminobenzidine, allowing an investigator to selectively excite and detect different probes present in the same section (Coons, 1961; Coons et al., 1941; Coons et al., 1942; Coons and Kaplan, 1950). A variety of fluorophores have been developed and are readily available for use in basic laboratory settings. More recently, other

reporter molecules such as quantum dots have been developed which have further increased the number of probes that can be accommodated on the basis of reduced spectral overlap (Byers and Hitchman, 2011; Chan et al., 2005; Chen et al., 2013; Dixon et al., 2015; Krenacs et al., 2010; Mansfield, 2017; Prost et al., 2016; Sweeney et al., 2008; Tholouli et al., 2008; Wu et al., 2003; Xu et al., 2013).

A major limitation to antibody combination for multiplex immunohistochemistry (mIHC) in settings without specialized equipment becomes immunocompatability given the cross-reactivity that can occur with antibodies raised in the same host species and of the same serogroup (Bogoslovsky et al., 2018; Krenacs et al., 2010). Limitations on immunocompatability can be obviated by different variations of sequential staining techniques (Dixon et al., 2015; Krenacs et al., 2010). Several groups have used tyramide signal amplification (TSA) and antibody elution to examine antibodies raised in the same species or serogroup (Buchwalow et al., 2018; Chao et al., 1996; Dixon et al., 2015; Lim et al., 2018; Mansfield, 2017; Pirici et al., 2009; Roy et al., 2019; Sorrelle et al., 2019; Stack et al., 2014; Wang et al., 1999; Zhang et al., 2017). TSA uses a peroxidase-mediated reaction to covalently deposit fluorophores to tyrosine side chains proximal to the target epitope (Lim et al., 2018; Wang et al., 1999). Because of the covalent bond, eluting primary and secondary antibodies following the TSA reaction will not affect the deposited fluorophores and allows the histologist to use conspecific antibodies in the same tissue section. Computational techniques to coregister images of the same tissue sections across multiple rounds of staining and signal stripping can also be used to generate mIHC data (Gerdes et al., 2013; Ma et al., 2017; Mansfield, 2017; Stack et al., 2014; Wegner et al., 2017). For both techniques, efficient and complete elution of primary and secondary antibodies is a critical step. As such, elution has become the focus of the present methodology study.

Several elution techniques have been utilized across several groups in formalin-fixed paraffin-embedded (FFPE) sections. Particularly in combination with TSA, heat-induced elution methods that recapitulate antigen retrieval steps are common (Ilie et al., 2018; Jufas et al., 2008; Krenacs et al., 2010; Lan et al., 1995; Lim et al., 2018; Mansfield, 2017; Parra et al., 2017; Roy et al., 2019; Saylor et al., 2018; Sorrelle et al., 2019; Stack et al., 2014; Wegner et al., 2017; Zhang et al., 2017). Typically, this technique is done using a microwave in citrate buffer at pH 6.0, however other methods have shown effectiveness (Krenacs et al., 2010; Lim et al., 2018; Sorrelle et al., 2019; Toth and Mezey, 2007; Zhang et al., 2017). Other groups have utilized low pH (2.0) solutions with sodium dodecyl sulfate (SDS) and glycine or lysine (Bolognesi et al., 2017; Buchwalow et al., 2018; Gendusa et al., 2014; Gut et al., 2018; Lan et al., 1995; Nakane, 1968; Narhi et al., 1997a; Pirici et al., 2009; Sorrelle et al., 2019; Wahlby et al., 2002). Older elution techniques originally developed for use with chromogenic mIHC utilize the inorganic salt

potassium permanganate ($KMnO_4$) and sulfuric acid (H_2SO_4) (Glass et al., 2009; Tramu et al., 1978), however these methods have not been utilized extensively with immunofluorescence. Additionally, there exist commercially available solutions designed for the elution step of mIHC (Buchwalow et al., 2018).

With varied reliability of the available elution techniques, approaches that denature rather than elute primary and secondary antibodies have been explored. β-mercaptoethanol (BME) reduces the disulfide bonds present in antibodies, thus breaking down their tertiary structure (Capel et al., 1980; Crivianu-Gaita et al., 2015). The use of BME has been applied to mIHC and has demonstrated excellent elution efficiency while maintaining antigenicity across multiple rounds of staining (Bolognesi et al., 2017; Cattoretti et al., 1993; Gendusa et al., 2014; Kim et al., 2012; Mansfield, 2017; van den Brand et al., 2014). Chaotropic salts such as guanidinium (GnHCl) have also been explored, as they efficiently denature antibody structure, however they require steps to recover antigen conformations following the denaturation step (Bolognesi et al., 2017; Gut et al., 2018; Narhi et al., 1997a; Narhi et al., 1997b). Furthermore, compared to BME, guanidinium shows less efficiency at eluting antibodies targeting dense, highly organized proteins such as many of the protein aggregates found in neurodegenerative diseases (Bolognesi et al., 2017).

Here, we examine and evaluate different multiplex histology methods with the goal to identify a reliable, low-cost method for routine use in the setting of a neuropathology laboratory studying neurodegenerative diseases. Additionally, we make recommendations on limitations, appropriate use, and necessary steps to incorporate techniques into experimental setups.

2. Material and Methods

A review of literature dating back to 1968 was used to select the elution strategies to be evaluated here. Following the literature review, two phases of experiments were implemented. The validation experiment examines the efficacy of a given elution technique for a specific antibody in one round of elution. Next, the tissue and antigen preservation was evaluated with the most reliable elution technique across multiple rounds of staining and elution in a variety of antibodies up to five rounds.

2.1 Literature review and elution technique selection

PubMed was searched with the terms “Antibody elution histology” and “Multiplex immunohistochemistry” with no date range set. “Antibody elution histology” yielded 462 results and “Multiplex immunohistochemistry” yielded 2021 results. In total, these combined searches yielded 2480 unique results ranging from November 1964 to May 2019. These results were manually filtered to

exclude irrelevant articles such as those related to immunotherapy, immunology, or methods applied to fluids or homogenates. From this, seven elution strategies were identified that described or utilized variations of protocols for multiplex immunohistochemistry with conspecific antibodies based on the use of (1) heat and a citrate buffer (Ilie et al., 2018; Jufas et al., 2008; Lan et al., 1995; Lim et al., 2018; Mansfield, 2017; Parra et al., 2017; Roy et al., 2019; Saylor et al., 2018; Sorrelle et al., 2019; Stack et al., 2014; Toth and Mezey, 2007; Wegner et al., 2017; Zhang et al., 2017); (2) a glycine/SDS solution at a low pH (Bolognesi et al., 2017; Gut et al., 2018; Nakane, 1968; Narhi et al., 1997a; Pirici et al., 2009; Sorrelle et al., 2019); (3) a commercially available denaturing solution (Buchwalow et al., 2018); (4) a low-pH oxidizing solution of $KMnO_4$ and H_2SO_4 (Glass et al., 2009; Tramu et al., 1978); (5) chaotropic salts (Bolognesi et al., 2017; Gut et al., 2018; Narhi et al., 1997a; Narhi et al., 1997b); (6) combining antibodies with drastically different abundances (Wang et al., 1999); and (7) BME denaturation (Bolognesi et al., 2017; Gendusa et al., 2014; Kim et al., 2012; Mansfield, 2017; van den Brand et al., 2014).

Chaotropic salts, such as guanidinium, do not reliably completely elute antibodies of highly aggregated, spatially organized targets (Bolognesi et al., 2017) so this technique was not tested here, as these are common features of neurodegenerative tauopathies. Utilizing strong oxidizers and low pH (Glass et al., 2009; Tramu et al., 1978) was excluded because strong oxidizing agents may inactivate fluorophores. The persistence of a TSA-amplified target throughout multiple rounds of elution may be desired to assist the coregistration of images from different staining rounds so this technique was also excluded. Lastly, the technique that relies on making the *a priori* assumption that some targets are in less abundance than others (Wang et al., 1999) is extremely limited in nature and based on tacit assumptions regarding the sensitivity of microscope cameras and variation in biological samples. This left four elution techniques depicted in Table 2.

2.2 Immunohistology

2.2.1 Tissue selection

Human brain tissue was collected from the Neurodegenerative Disease Brain Bank at the University of California, San Francisco's Memory and Aging Center. 8µm thick FFPE sections were mounted on Ultra Bond adhesive slides (SL6023-1, Avantik BioGroup). All sections were incubated in an oven at 65^{o}C for at least 18 hours. The post-mortem interval was 7.6 hours for the CP13 test slides, 8.2 hours for the NeuN test slides, and 30.3 hours for the GFAP test slides. Samples were fixed in 10% neutral buffered formalin

for 72 hours. The collection of these tissues was approved by the institutional review boards at the University of California, San Francisco.

2.2.2 Primary antibodies

For the initial validation experiments, CP13 (1:800; gift of Peter Davies), a marker of phospho-Ser202 tau, was used for every elution protocol tested as it represents a commonly used monoclonal antibody that targets a dense, highly organized target, pathologic aggregates. NeuN (1:600; #266004, Synaptic Systems) and GFAP (1:1800; #ab68428, Abcam) were also used as examples of common markers of cell-type. Table 1 depicts the host species, vendor information, and dilution used for all primary antibodies used in this study, including those used in the tissue and antigen preservation experiments.

2.2.3 Validation experiments

The four selected candidate elution techniques were examined in three antibodies representing two cell markers and a commonly used marker for neurodegenerative tauopathy. TSA was utilized at three steps as the reporter in this protocol to evaluate (1) presence, distribution, and proper retrieval and development of antigen; (2) full elution; and (3) preservation of antigen after elution treatment.

Slides underwent serial deparaffinization steps as follows: three ten-minute immersions in xylene, two two-minute immersions in 100% ethanol, two two-minute immersions in 96% ethanol, and one two-minute immersion in 80% ethanol. Slides were then immersed in a solution of 3% hydrogen peroxide (H_2O_2) and 80% methanol for 30 minutes to quench any endogenous peroxidase. Sections underwent three two-minute washes in distilled water (dH_2O) before being transferred into a 10% solution of 0.1M citrate buffer with 0.05% tween-20 for antigen retrieval. In the antigen retrieval solution, sections were cycled through an autoclave set at 151^{o}C at the five-minute setting. Following antigen retrieval, sections were left to cool to room temperature (RT) for approximately 60 minutes. After thorough washing in a solution of 1x PBS and 0.05% tween (PBST), sections were immersed in a solution of 5% milk with 0.05% tween (herein referred to as milk) for 30 minutes. Sections were then incubated in CP13 (1:800, gift of Peter Davies), NeuN (1:600; #266004, Synaptic Systems), or GFAP (1:1800; #ab68428, Abcam) in the milk solution for 16 hours overnight at RT.

After washing in PBST, sections were incubated in a 1:400 concentration of goat-α-mouse IgG(H+L) HRP-conjugated secondary antibody (R-05071, Advansta), goat-anti-guinea pig IgG (H+L) HRP conjugated-secondary antibody (R-05076, Advansta), or goat-anti-Rabbit IgG (H+L) HRP conjugated-secondary antibody (R-05072, Advansta) diluted in PBST for 60 minutes. Following additional PBST

washes, the antibodies were developed with TSA following the manufacturer's instructions with a solution of 1:100 Alexa Fluor 647 (AF647) Tyramide (B40958, Thermo Fisher) and 1:100 of 100x H_2O_2 in 1x tris-buffered saline. This amplification occurred for 15 minutes and was followed by PBST washes. As there was no need to inactivate the HRP in this experiment, we omitted a 30 minute incubation in a solution of 5% sodium azide (NaN_3) in 3% H_2O_2 designed to inactivate the HRP (Ortiz de Montellano et al., 1988). Following TSA, one of several possible elution techniques was employed according to the protocols given by the references noted in Table 2.

In order to determine the effectiveness of the elution technique, sections were re-incubated in a 1:400 concentration of goat-α-mouse IgG(H+L) HRP-conjugated secondary antibody (R-05071, Advansta), goat-anti-guinea pig IgG (H+L) HRP conjugated-secondary antibody (R-05076, Advansta), or goat-anti-Rabbit IgG (H+L) HRP conjugated-secondary antibody (R-05072, Advansta) diluted in PBST for 60 minutes. After PBST washing, an additional TSA step with Alexa Fluor 546 (AF546) Tyramide (B40954, Thermo Fisher) for 15 minutes. The TSA step was ended with a PBST wash. In order to determine if antigenicity was preserved, an additional incubation in CP13 (1:800, gift of Peter Davies) diluted in milk was done for 16 hours overnight at RT.

Again, after washing in PBST, sections were incubated in a 1:400 concentration of goat-α-mouse IgG(H+L) HRP-conjugated secondary antibody (R-05071, Advansta), goat-anti-guinea pig IgG (H+L) HRP conjugated-secondary antibody (R-05076, Advansta), or goat-anti-Rabbit IgG (H+L) HRP conjugated-secondary antibody (R-05072, Advansta) diluted in PBST for 60 minutes. Following additional PBST washes, the antibodies were developed with TSA following the manufacturer's instructions with a solution of 1:100 Alexa Fluor 488 (AF488) Tyramide (B40953, Thermo Fisher) and 1:100 of 100x H_2O_2 in 1x tris-buffered saline. This amplification occurred for 15 minutes and was followed by PBST washes.

Following PBST washes and a transfer through 70% ethanol, the sections were treated with a solution of 0.8% Sudan Black-B in 70% ethanol for 35 minutes to block for lipofuscin. After 35 minutes, two ten-second washes in 70% ethanol was used to remove excess Sudan black-B. Sections were re-hydrated in PBS then coverslipped with Prolong Glass Antifade Mountant with NucBlue (P36981, Thermo Fisher).

Different TSA-deposited fluorophores represented different outcomes here. Because TSA covalently deposits fluorophores to the tissue, fluorescent signal will remain following the elution of the antibody (Lim et al., 2018; Wang et al., 1999). For this reason, AF647 signal represents the distribution of antibody

reactivity independent of the elution, thus the signal in this channel (Cy5) becomes the standard that other channels are compared to. After the elution technique and re-incubation in HRP-conjugated secondary, no primary or HRP-conjugated secondary antibody should be present. An attempt to deposit AF546-tyramide at this step should yield no signal in the DsRed channel if the elution was successful. Overlapping signal between AF546 (visualized in the DsRed channel) and AF647 (visualized in the Cy5 channel) would indicate that the antibody complex was not successfully eluted. Finally, re-incubation in the same primary antibodies followed by the HRP-conjugated secondary antibodies and AF488-tyramide amplification represents the preservation of antigenicity. If there is lack of overlap between AF488 (visualized in the GFP channel) and AF647 (visualized in the Cy5 channel), the elution technique likely degraded the antigen of interest.

2.2.4 Tissue and antigen preservation

Incubation in BME was the only technique that successfully eluted all antibodies and did not cause dissociation of the tissue from the slide. We next explored whether BME-based elution could be used in a setup that would accommodate multiple rounds of staining, imaging, eluting up to five rounds. Dissociation of the section from the slide and/or markedly decreased signal for a given antibody represents the upper limit of multiplex histology using BME-based elution. In a multiplex setup, an investigator would likely want to utilize a marker that persists through each round that could be used for computer-assisted coregistration of target signals across different rounds. Here, we use TSA to visualize the distribution of an antigen for neurons, NeuN, across multiple rounds, as well as the nuclear marker, Hoechst 33342.

Five serial sections were allocated for each of the primary antibody groups (each group consisting of one primary antibody raised in mouse and one in rabbit from Table 1). Slides underwent serial deparaffinization steps as follows: three ten-minute immersions in xylene, two two-minute immersions in 100% ethanol, two two-minute immersions in 96% ethanol, and one two-minute immersion in 80% ethanol. Slides were then immersed in a solution of 3% hydrogen peroxide (H_2O_2) and 80% methanol for 30 minutes to quench any endogenous peroxidase. Sections underwent three two-minute washes in distilled water (dH_2O) before being transferred into a 10% solution of 0.1M citrate buffer with 0.05% tween-20 for antigen retrieval. In the antigen retrieval solution, sections were cycled through an autoclave set at 151^{o}C at the five-minute setting. Following antigen retrieval, sections were left to cool to room temperature (RT) for approximately 60 minutes. After thorough washing in a solution of 1x PBS and 0.05% tween (PBST), sections were immersed in a solution of 5% milk with 0.05% tween (herein referred to as milk) for 30 minutes. Sections were then incubated in a primary antibody cocktail

consisting of one antibody raised in mouse, one antibody raised in rabbit, and NeuN (1:600 dilution, 266 004, Synaptic Systems) in the milk solution for 16 hours overnight at RT.

After primary incubation, slides were washed thoroughly in PBST then incubated in 1:400 goat-anti-guinea pig IgG (H+L) HRP conjugated-secondary antibody (R-05076, Advansta) diluted in PBST for 60 minutes. Following additional PBST washes, the antibodies were developed with TSA following the manufacturer's instructions with a solution of 1:100 AF647 Tyramide (B40958, Thermo Fisher) and 1:100 of 100x H_2O_2 in 1x tris-buffered saline. The reaction was stopped with a PBST wash.

Following PBST washes and a transfer through 70% ethanol, the sections were treated with a solution of 0.8% Sudan Black-B in 70% ethanol for 35 minutes to block for lipofuscin. After 35 minutes, two 10-second washes in 70% ethanol was used to remove excess Sudan black-B.

After rehydration in PBS, sections were incubated in a cocktail of 1:400 Goat anti-Rabbit IgG (H+L) (A-11008, Life Technologies) and 1:400 AF546 Goat anti-Mouse IgG (H+L) (A-11003, Life Technologies) in PBS for 90 minutes. Following washing in PBS, the slides were coverslipped with Prolong Glass Antifade Mountant with NucBlue (P36981, Thermo Fisher) then imaged at 10x. After imaging, the slides were submerged in PBST overnight and agitated in order to remove the coverslip. Sections were washed in additional PBST to remove any excess mounting media.

BME elution solution was prepared according to Gendusa et al. (2014). Under a hood, 20ml of 10% SDS was mixed with 12.5ml of 0.5M Tris-HCl (pH 6.8), 67.5ml of deionized (Millipore) water, and 0.8ml of BME. The solution was heated to 56^{o}C prior to use. Sections were incubated in the heated BME solution for 30 minutes. Sections were washed in four 15-minute dH_2O immersions. Sections were then washed in TBST for five minutes and then re-blocked in milk solution for 30 minutes. From each antibody group, one slide is incubated overnight in the milk solution to represent the elution control. All other slides receive the same primary antibody cocktail as the first night for an overnight incubation at RT.

After the overnight incubation in the primary antibodies, all slides were washed thoroughly in PBST. The protocol beginning with the Sudan Black blocking was repeated another five times, each time removing one slide and keeping it coverslipped to represent the elution control for that round. A flowchart depiction of this experiment is found in Figure 1.

2.2.5 Microscopy and imaging

Slides were imaged on a Zeiss AxioImager.A2 microscope equipped with a Zeiss Colibri 7:Type FR-R[G/Y]CBV-UC 7-channel fluorescence light source. NucBlue (Hoechst 33342) was visualized with a DAPI filter set, AF488 visualized with a GFP filter set, AF546 with a DsRed filter set, and AF647 with a Cy5 filter set.

3. Results

3.1 Phosphorylated tau inclusions

For slides stained for CP13, colocalization was noted between AF546 and AF647 for low-pH/glycine-based and denaturing solution-based elution, indicating that there was only partial elution of the primary and/or secondary antibodies. For BME-based and microwave-based elution, there was no overlap between AF546 and AF647 indicating that there was highly efficient elution of CP13 and/or the goat-α-mouse IgG(H+L) HRP-conjugated secondary antibody. In all sections, there was nearly complete colocalization between AF488 and AF647, indicating that antigenicity of the phospho-Ser202 Tau epitope detected by CP13 was preserved through each of the tested elution techniques in the one round of elution. In fact, more signal is noted coming from AF488 with the microwave elution and glycine/SDS elution, suggesting that these strategies expose additional epitopes for antibody reactivity. Furthermore, tissue did not dissociate from the glass for all elution techniques with the exception of the heat-driven elution step. In just the one round of microwave elution, the tissue sections tended to dissociate from the slide following the microwave treatment. A summary of these results is depicted in Table 3 and Figure 2.

3.2 Cell-type markers

For slides stained for NeuN or GFAP, no colocalization was noted between AF546 or AF647 for any of the elution strategies, indicating that there was efficient elution of these antibodies and/or the HRP-secondary antibodies. In all sections, there was nearly complete colocalization between AF488 and AF647, indicating that the antigenicity of the epitopes for these antibodies is preserved through these techniques. In fact, more signal is noted coming from AF488 in all strategies tested, suggesting that the elution reagents additional epitopes for antibody reactivity, however, as this was noted in all strategies, this may also be due to increased autofluorescence and camera sensitivity in the GFP channel. Sections moderately dissociated from slides with the microwave elution treatment. A summary of these results is depicted in Table 3 and Figures 3 and 4.

3.3 Tissue and antigen preservation across multiple rounds

In the tissue and antigen preservation experiment, antigenicity was preserved throughout all rounds where tissue remained intact, indicating that BME has little effect on epitope conformation for those targets checked. The elution control slides confirmed that BME effectively eluted each antibody in each round. The TSA-deposited AF647, marking NeuN, maintained signal throughout all rounds despite only immunostaining for NeuN in the first round. Similarly, inclusion of Hoechst 33342 signal was noted in each round after coverslipping using Prolong with NucBlue. By the fifth round of staining, tissue sections had begun to dissociate from the glass slide.

4. Discussion

In this study, we examine the potential of several previously reported techniques for antibody elution in the context of a pipeline for neurodegenerative disease research in human post-mortem tissue. We found that the use of heat, low pH with glycine, and a commercial denaturing solution did not completely elute CP13 in a test using TSA to report results. All tested techniques maintained epitope integrity, however the heat-based elution strategy used was damaging to the tissue sample.

Microwave heating of samples in citrate buffer is the recommended strategy for elution from ThermoFisher Scientific (Company publication #MAN0015834) and PerkinElmer ("Opal Multiplex IHC Assay Development Guide") for use with TSA mIHC. This method is widely used and works to elute most antibodies (Ilie et al., 2018; Jufas et al., 2008; Lan et al., 1995; Lim et al., 2018; Mansfield, 2017; Parra et al., 2017; Roy et al., 2019; Saylor et al., 2018; Sorrelle et al., 2019; Stack et al., 2014; Toth and Mezey, 2007; Wegner et al., 2017; Zhang et al., 2017). In our test, we used CP13, a mouse monoclonal antibody raised against human phospho-Serine-202 tau (Andorfer and Davies, 2000). In tissue from patients of tauopathies, CP13 will stain neurofibrillary tangles and paired helical filaments which are highly aggregated, organized, dense protein structures composed of tau (Brion, 1998). These factors may contribute to the inability of this technique, as well as low pH with glycine and commercial denaturing solution, to not completely elute CP13, as would be the predicted the case with chaotropic salts (Bolognesi et al., 2017). Notwithstanding this limitation and the frequent dissociation of the tissue from the slide in the microwave, it remains a quick and effective technique for the elution of many antibodies.

The tissue and antigenicity preservation experiments implemented here examined the feasibility of using BME-based elution across several rounds of staining, eluting antibodies, and theoretically restaining for other antibodies to allow for computer-assisted coregistration of many target signals. Here, we show that BME does not noticeably alter target signal up to five rounds of staining and eluting. Additionally, we show that the use of TSA reporting of a target or Hoechst 33342 to establish microscopic landmarks for

coregistration of images in different rounds is feasible with BME. There were issues regarding tissue integrity in some samples, though. These issues may be ameliorated by improvements to histological processing, although this may represent a ceiling for the capabilities of this kind of technique. Nevertheless, if using three new antibodies from different species in each round, a realistic parameter for most fluorescence microscopy setups, one could analyze up to 15 targets in a single section based off these results. In a setup of this type, an investigator would necessarily optimize the parameters of each individual antibody following the kind of treatment sections would be subject to. For example, if an antibody is to be used following three rounds of elution, one should determine the antibody concentration in tissue samples that received three treatments of BME.

In the absence of more scarce techniques such as Matrix Assisted Laser Desorption/Ionization (Michno et al., 2018), PCR-based target quantification (Derrieux et al., 2019), mass cytometry (Bendall et al., 2011), and other commercial non-destructive spatial profiling methods (Merritt et al., 2019), this pipeline becomes a valuable framework for traditional histopathology settings, particularly when incorporating automated image analysis tools (Alegro et al., 2017; Camp et al., 2002; Marrero et al., 2016; McCabe et al., 2005).

In the framework of the aforementioned ideal pipeline for studying molecular pathology in neurodegenerative diseases, elution strategies should be carefully examined for each antibody and tissue type as a key part of the probe validation phase. A main limitation of our study is the limited set of antibodies evaluated. While BME worked ubiquitously in the antibodies studied here, the need for careful validation is evident when considering the abundance of studies using strategies that were not completely effective here (Bolognesi et al., 2017; Gendusa et al., 2014; Gown et al., 1986; Gut et al., 2018; Ilie et al., 2018; Jufas et al., 2008; Lan et al., 1995; Lim et al., 2018; Mansfield, 2017; Nakane, 1968; Narhi et al., 1997a; Narhi et al., 1997b; Parra et al., 2017; Pirici et al., 2009; Roy et al., 2019; Saylor et al., 2018; Sorrelle et al., 2019; Stack et al., 2014; Toth and Mezey, 2007; Tramu et al., 1978; van den Brand et al., 2014; Wahlby et al., 2002; Wegner et al., 2017; Zhang et al., 2017). These strategies should not be completely dismissed, though, as they may save time, have varied effectiveness for certain antibodies, and, if effective, may avoid the use of BME, a fatally toxic substance. Here, the method used in the validation experiments may serve as a helpful guideline for testing elution strategies prior to implementation in mIHC. By utilizing TSA, our reporter is extremely sensitive to residual antibodies left after elution steps that might otherwise be missed if using traditional immunofluorescence (Chao et al., 1996; Wang et al., 1999). Furthermore, elution control slides should be routinely implemented in mIHC experiments in the same manner that it was implemented in the tissue and antigen preservation

experiment here. With proper validation, optimization, and implementation of controls, use of mIHC is realistic in standard histopathology labs and will likely be key for elucidating the molecular fundamentals of neurodegenerative diseases.

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5.2 Disclosures

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6. Tables

Table 1: Primary antibodies used in this study

Antibody	Host	Source	Working dilution
CP13	Ms, monoclonal IgG_{2b}	Peter Davies	1:800
TMEM119	Rb, polyclonal	Sigma Aldrich (HPA051870)	1:500
PHF-1	Ms, monoclobal IgG_1	Peter Davies	1:9000
GFAP	Rb, monoclonal EPR1034Y	Abcam (ab68428)	1:1800
MC1	Ms, monoclobal IgG_1	Peter Davies	1:1000
T18	Rb, polyclonal	Rakez Kayed	1:1500
NeuN	GP, polyclonal	Synaptic Systems (266 004)	1:600

Table 2: Elution techniques tested for validation experiments

Strategy	Description	Reference for protocol
Heat	Microwave in citrate buffer with 0.05% tween. High setting until boiling then 15 minutes at 20% (Note: referenced paper suggests 5 minutes at 50%).	Toth and Mezey (2007)
Low pH with glycine	Glycine mixed with SDS, at pH 2.0 and heated to 50^{o}C for 30 minutes.	Pirici et al. (2009)
Denaturing solution	One part of solution A to two parts of solution B from the Denaturing Solution Kit from Biocare Medical	Manufacturer instructions (part DNS001L)
BME	Tris-HCl mixed with SDS and BME, heated to 56C and incubated for 30 minutes. Washed in TBS.	Gendusa et al. (2014)

Table 3: Summary of outcomes of various elution techniques.

Strategy	Effective elution			Antigenicity preservation			Intact tissue		
	CP13	*NeuN*	*GFAP*	*CP13*	*NeuN*	*GFAP*	*CP13*	*NeuN*	*GFAP*
Heat	+	+	+	+	+	+	-	-	-
Low pH with glycine	-	+	+	+	+	+	+	-	-
Denaturing solution	-	+	+	+	+	+	+	+	+
BME	+	+	+	+	+	+	+	+	+

7. Figures

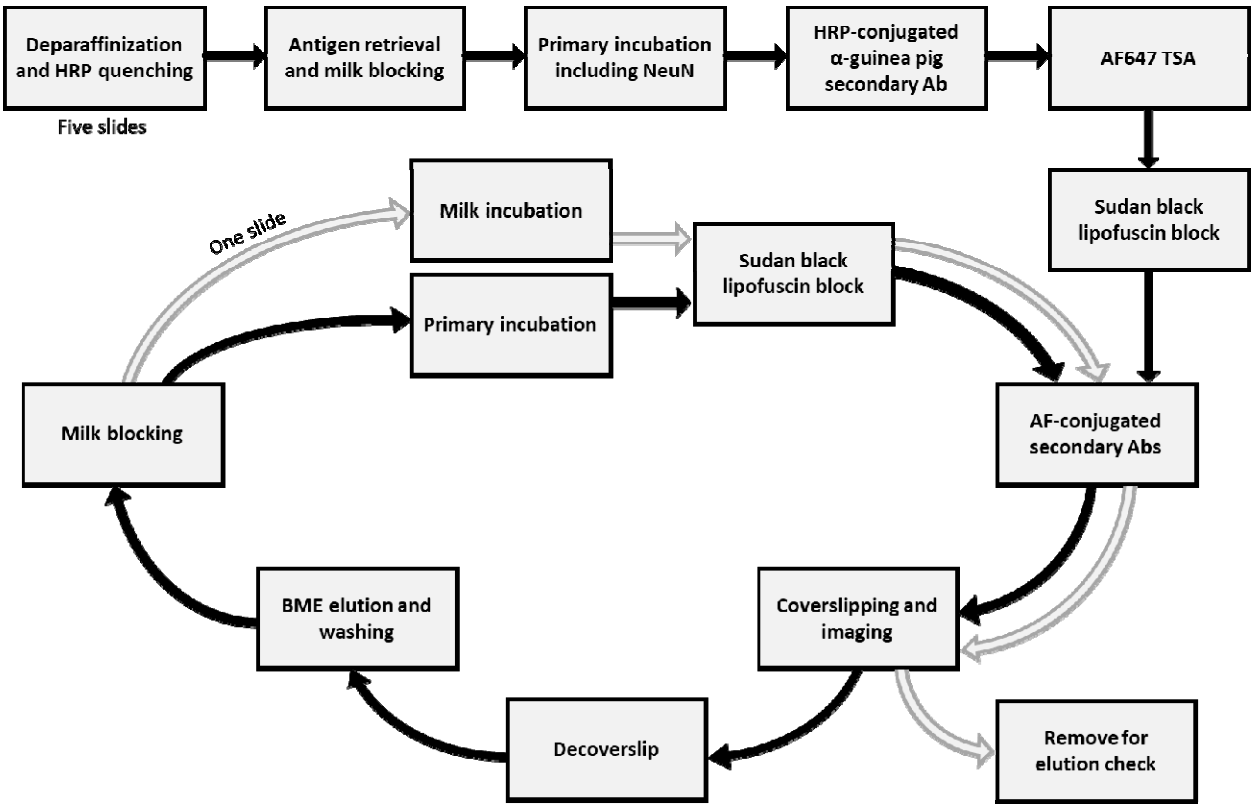

Figure 1: Flowchart depiction of tissue and antigen preservation experiment with each antibody set starting with five slides. One slide is removed each round for elution checks and then kept in storage.

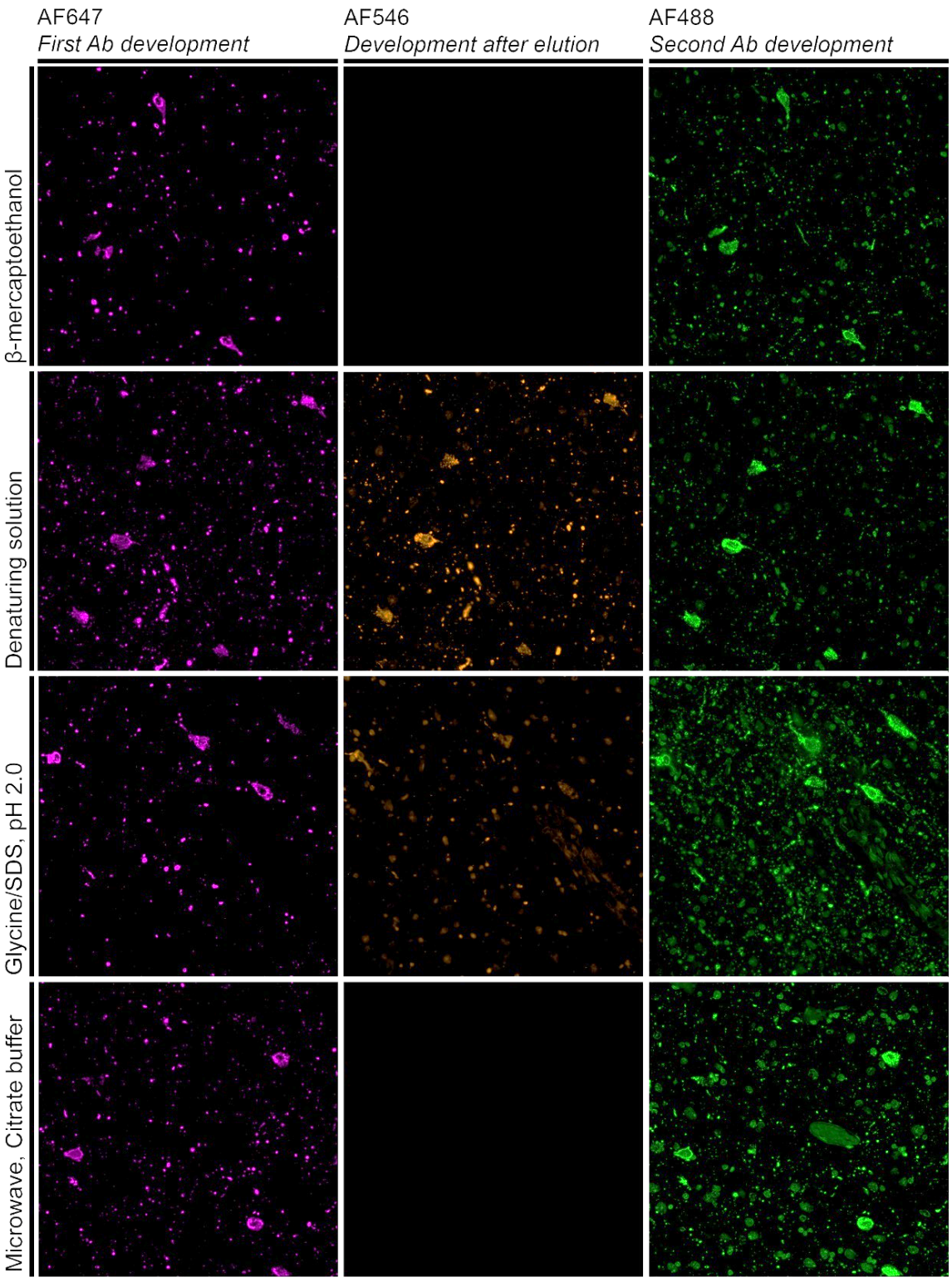

Figure 2: Photomicrographs of the validation experiments for CP13. Successful elution and antigen preservation is represented by overlapping signal from AF647 and AF488 with zero signal from AF546 (β-mercaptoethanol and Microwave). The denaturing solution resulted in significant signal from AF546 and the Glycine/SDS, pH 2.0 resulted in decreased, but noticeable signal from AF546.

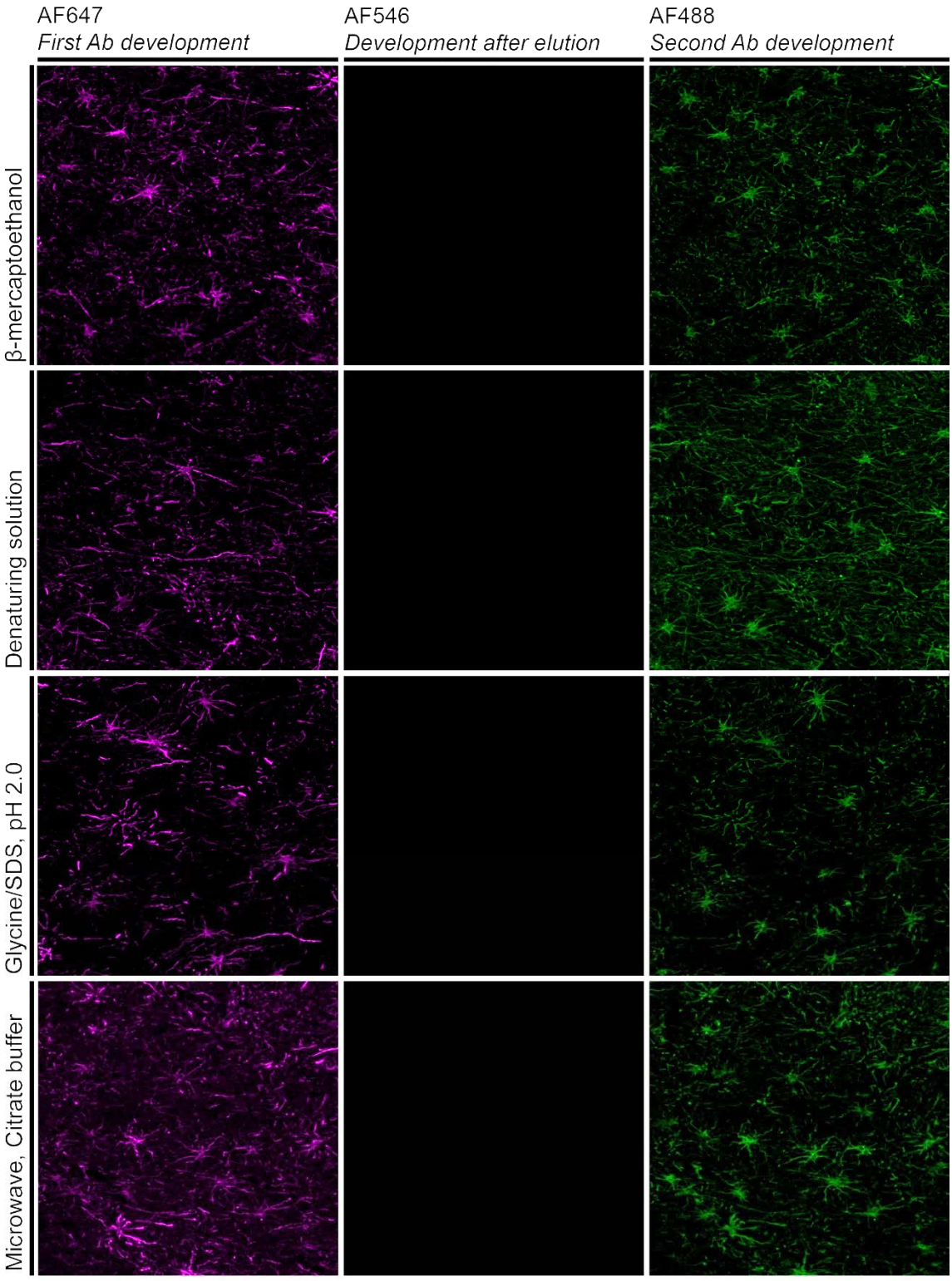

Figure 3: Photomicrographs of the validation experiments for GFAP. Successful elution and antigen preservation is represented by overlapping signal from AF647 and AF488 with zero signal from AF546 for all tested elution strategies.

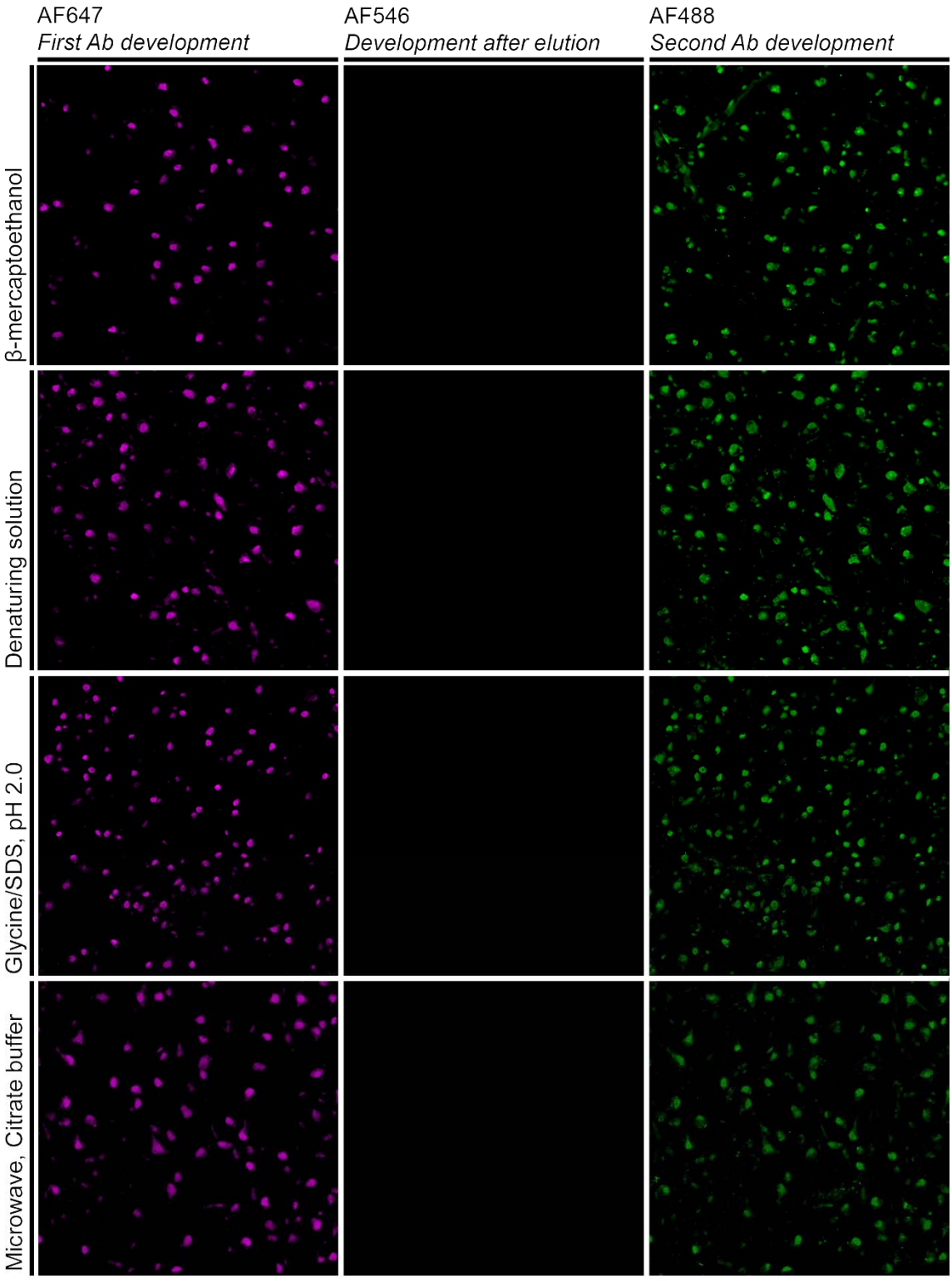

Figure 4: Photomicrographs of the validation experiments for NeuN. Successful elution and antigen preservation is represented by overlapping signal from AF647 and AF488 with zero signal from AF546 for all tested elution strategies.

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