Multiplex immunofluorescence methods in neurodegenerative diseases

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Abstract

BACKGROUND: The ability to routinely visualize multiple targets within human tissue sections would markedly facilitate the discovery and validation of molecular pathways underlying neurodegenerative diseases. Several techniques have been proposed, but few have been thoroughly evaluated in the context of human tissue in neurodegenerative diseases associated with tau protein deposition.

NEW METHOD: We reviewed and evaluated several established techniques for multiplex immunohistochemistry and immunofluorescence and created a pipeline tailored for the intricacies of human brain postmortem tissue. We also make recommendations on appropriate use and necessary validation steps for implementing multiplex immunofluorescence methods in the setting of neuropathology laboratories focused on neurodegenerative diseases without the need of sophisticated equipment.

RESULTS: The proposed protocol enables reliable primary and secondary antibody elution and minimize the odds of cross reactivity. Out of all tested methods, β-mercaptoethanol reliably eluted all antibodies tested and maintained antigen integrity.

COMPARISON WITH EXISTING METHODS: Several techniques for multiplex immunohistochemistry have been widely used in the literature, however few experiments explicitly validate elution parameters in the specific experimental setup. Here, we illustrate the necessity of this validation, as well as examine the longevity of tissue samples and antigens across five rounds of immunostaining.

CONCLUSIONS: Multiplex immunofluorescence protocols for studying neurodegenerative conditions in postmortem human studies are feasible and can be implemented in laboratories lacking sophisticated equipment. Nevertheless, the inclusion of elution parameters in the optimization and validation phase of any experiment is prudent. Furthermore, elution control slides should be incorporated into multiplex immunohistology experiments that rely on effective elution of antibodies during any step.

Keywords

Multiplex histology, Immunohistochemistry, Immunofluorescence, Neuropathology

1. Introduction

Given the selective vulnerability underlying neurodegenerative diseases (Fu et al., 2018), one of the biggest hurdles to elucidating fundamental biology and etiology is the difficulty involved in developing models that recapitulate the full array of disease processes. Even the best-probed molecular pathways 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.

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) 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. Computing 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 elution of primary and secondary antibodies is a critical step.

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), however these methods have not been utilized extensively with immunofluorescence (Glass et al., 2009; Tramu et al., 1978). 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

Two phases of experiments were implemented for this study. The validation experiment examines the efficacy of a given elution technique for a specific antibody in one round of elution. The longevity experiment tests how many rounds of staining and elution a section can undergo for a given technique in a variety of antibodies up to five rounds.

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 and the Biobank for Aging Studies at the University of São Paulo. 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 collection of these tissues was approved by the institutional review boards at the University of California, San Francisco.

2.2 Primary antibodies

For the initial validation experiments, CP13 (1:800, gift of Peter Davies) was used for every elution protocol tested as it represents a commonly used monoclonal antibody that targets a dense, highly organized target, pathologic tau aggregates. Other antibodies listed in Table 1 were also tested in the validation experiments, however, as they were not used with all elution techniques tested, we will only

report the methods and results for those validation tests done with CP13. Table 1 depicts the host species, vendor information, and dilution used for all primary antibodies used in this study.

2.3 Validation experiments

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^oC 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) 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) 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) 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) 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).

2.4 Longevity experiment

Five serial sections were allocated for each of the primary antibody groups. 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.

2.5 Microscopy and imaging

Slides were checked and 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

For all tested elution techniques, there was little or no overlap between AF546 and AF647 indicating that there was highly efficient elution of CP13 and the goat-α-mouse IgG(H+L) HRP-conjugated secondary

antibody. With the exception of BME, some, albeit infrequent, colocalization was noted between AF546 and AF647, indicating that there was only partial elution of the primary and/or secondary antibodies. No colocalization was detected between AF546 and AF647 in the section used to test elution by BME. 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. Furthermore, tissue integrity was maintained for all elution techniques with the exception of the heat-driven elution step. In just the one round of elution, the tissue sections tended to dissociate from the slide following the microwave treatment. A summary of these results is depicted in Table 3.

In the longevity experiment, antigenicity was preserved throughout all five rounds, 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. By the fifth round of staining, several tissue sections had partially lost integrity and began to dissociate from the glass slide. The TSA-deposited AF647, marking NeuN, maintained signal throughout all rounds despite only immunostaining for NeuN in the first round.

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 longevity 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 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 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 longevity 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 with CP13

Strategy	Effective elution	Preserved antigenicity	Tissue integrity
Heat	Partial	Yes	Poor
Low pH with glycine	Partial	Yes	Good
Denaturing solution	Partial	Yes	Good
BME	Yes	Yes	Good

7. Figures

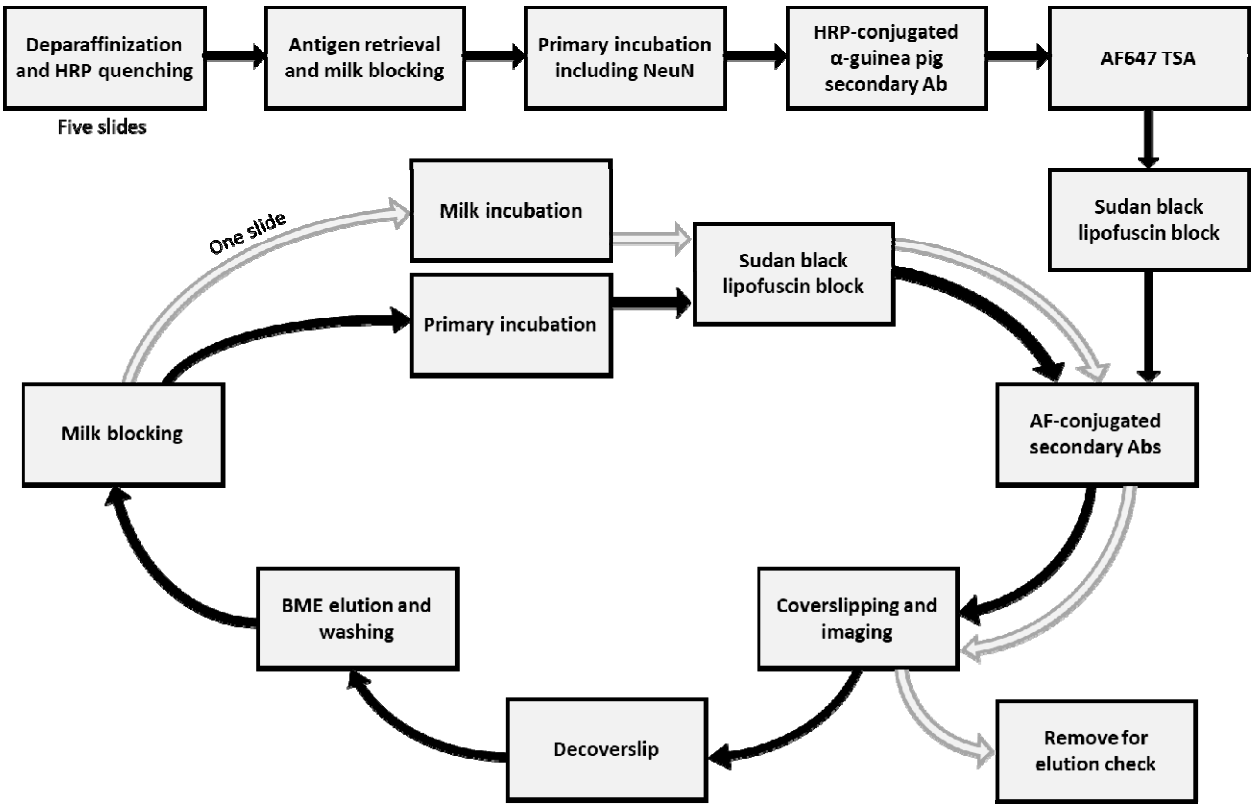

Figure 1: Flowchart depiction of longevity experiment with each antibody set starting with five slides. One slide is removed each round for elution checks and then kept in storage.

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