Multiplex immunofluorescence methods in neurodegenerative diseases

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Abstract

The application of multiplex immunofluorescence to human post-mortem tissue would drive observational studies of selective vulnerability in neurodegenerative proteinopathies. Efficient elution of antibodies is critical for flexibility of antibody combinations and the ability to utilize a sample in multiple rounds of immunostaining. Here, we test two elution strategies for antibodies relevant to the study of selective vulnerability in neurodegenerative diseases in post-mortem human samples from both long-fixed and short-fixed tissue. Both 2-Mercaptoethanol/SDS-based and Urea/SDS/Glycine-based elution strategies work well with the antibodies selected, confirming observations from previous studies with other antibody types.

Introduction

Given the selective vulnerability underlying neurodegenerative diseases (Fu, Hardy et al. 2018), one of the biggest hurdles to elucidating the fundamental biology and basic etiology is the difficulty involved in developing models that recapitulate the full array of disease processes. Overcoming these hurdles can be aided using human post-mortem tissue, thus allowing for direct observation of different proteinopathies and the classification of different cell-types. Applying different biochemical methods with traditional histopathology allows for the demonstration of several antigens without the hinderance of antibody cross-reactivity and restricted number of fluorescence filters that can be used in combination (Mason, Micklem et al. 2000).

Completely eluting antibodies from their antigens is paramount for successful and flexible multiplex histology. A variety of techniques have been proposed to elute antibodies ranging from the application of heat and extreme pH with the objective to break the antibody-antigen interactions. Unfortunately, many of these techniques can be destructive to the tissue samples. Furthermore, extreme pH and temperature do not reliably elute antibodies (Wang, Singh et al. 2007, Argentieri, Pilla et al. 2013, Gendusa, Scalia et al. 2014). An ideal technique would selectively and efficiently strip antibodies from their respective epitopes, prevent them from re-binding to those epitopes, and protect tissue and epitope integrity.

From the wide array of strategies developed in biochemistry for techniques such as antibody purification from agarose beads and nitrocellulose, we selected a combination of a urea/glycine/SDS-based technique and a 2-mercaptoethanol/SDS-based technique to attempt multiple rounds of staining. These techniques have been previously tested in human formalin-

fixed paraffin-embedded (FFPE) tissue (Cattoretti, Pileri et al. 1993, Pirici, Mogoanta et al. 2009, Gendusa, Scalia et al. 2014), however they have not been validated in the antibodies useful for investigating selective vulnerability in neurodegenerative diseases. Here, we test these methods with antibodies either used to distinguish nervous system cell types or different proteinopathies in both short-fixed (<1 month) and long-fixed human FFPE tissue.

Materials and Methods

Samples

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. With the exception of cases stained for PH8 or TH, FFPE sections were sampled from the middle frontal gyrus from the brains of patients with a post-mortem diagnosis of Alzheimer's disease. For cases stained for PH8 or TH, FFPE sections were sampled from the pons of patients with a Braak stage of I-II and no other co-pathologies. For the purposes of this study, cases were de-identified with the exception of their post-mortem diagnosis. For cell-type markers, both short-fixed (<1 months fixation) and long-fixed tissue (1-3 years fixation) were examined. All sections were incubated in an oven at 65ºC for at least 18 hours. The collection of these tissues by these repositories is approved by their respective institutional review boards.

Antibodies

All primary antibodies used in this study are displayed in Table 1. Antibodies were selected to represent the kinds of proteinopathies and cell-type markers that would be used to study neurodegenerative diseases. Working dilutions were determined following individual optimizations.

Table 1: Primary antibodies used in this study

Antibody	Host species	Source	Working dilution
CP13	Ms	Peter Davies (Dept. of Pathology, Albert Einsten College of Medicine)	1:800
T231	Ms	R&D Systems (MAB34941-100)	1:3000
GFAP	Rb	Abcam (Ab68428)	1:1800
TMEM119	Rb	Sigma Aldrich (HPA051870)	1:500
PH8	Ms	Millipore (MAB5278)	1:1000
TH	Rb	Millipore (AB5935)	1:1000

Immunofluorescence

Sections were deparrafinized in three 10-minute washes of xylene followed by 2x2 minutes in 100% ethanol, 2x2 minute washes in 96% ethanol, and 1x2 minute wash 80% ethanol. Endogenous peroxidase was then blocked in a 30 minute wash in 3% H_2O_2 in methanol. Sections were then washed in dH_2O before antigen retrieval. Antigen retrieval occurred in a 10% solution of 0.1M Citrate Buffer with 0.05% tween with an autoclave set at 151ºC at the 5-minute setting. Following antigen retrieval, sections were left to cool to room temperature (RT) for roughly 60-minutes and were then washed thoroughly with 1x PBS with 0.05% tween (PBST). Blocking occurred using 5% nonfat milk with 0.05% tween for 30 minutes and was then followed by an

overnight (16-hour) incubation in the primary antibody at RT. The primary antibodies were diluted to the appropriate working dilution (Table 1) with the milk/tween solution.

Following the overnight incubation, sections were washed with PBST. This was followed by a 35 minute incubation in an HRP-conjugated secondary antibody diluted to 1:400 in PBST. The HRP-conjugated secondary was matched to the host species of the primary antibody. The Goat-α-rabbit IgG(H+L) HRP-conjugated secondary antibody (R-05072) and Goat-α-mouse IgG(H+L) HRP-conjugated secondary antibody (R-05071) were both sourced from Advansta. The secondary antibody incubation was followed by PBST washes and then a tyramide signal amplification with Alexa Fluor 647 Tyramide (B40958, Thermo Fisher). This amplification occurred for 15 minutes and was followed by PBST washes to stop the reaction. This amplification was followed by the appropriate elution step. For each antibody, both elution steps were tested on separate slides. For those antibodies with both short-fixed and long-fixed tissue, each fixation-time/antibody was tested with both elution steps on separate slides.

Following the elution steps, sections were incubated with the same HRP-conjugated secondary antibody at 1:400 in PBST for 35 minutes. This secondary antibody incubation was followed by PBST washes and then a tyramide signal amplification with Alexa Fluor 546 Tyramide (B40954, Thermo Fisher) for 15 minutes and ended with PBST washes. This amplification step allows us to determine if the originally deposited antibody complex was sufficiently eluted. The sections were then re-incubated overnight at RT with the same primary antibody as tested on the first night in order to determine if antigenicity was affected by the elution steps.

Following the overnight incubation in the primary antibody solution, sections were again incubated in a 1:400 solution of an HRP-conjugated secondary antibody for 35 minutes. This was followed by another tyramide signal amplification with Alexa Fluor 488 Tyramide (B40953, Thermo Fisher) for 15 minutes. Following PBST washes and a transfer through 70% ethanol, the sections were treated with 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. Sections were re-hydrated in PBS then coverslipped with Prolong Glass Antifade Mountant with NucBlue (P36981, Thermo Fisher).

Elution

For the urea/Glycine/SDS elution step, 6g of urea was dissolved in 100mL of dH_2O and heated to 95ºC for 10 minutes. After this pre-heating, sections were placed in the urea solution and incubated for 15-minutes at 95ºC. These sections were then washed in 1x TBS with 0.05% tween (TBST) for 1-hour. This was followed by a wash in SDS-glycine at pH 2.01 at 55ºC for 30 minutes following the protocol in Pirici, Mogoanta et al. (2009). This was followed by another 1-hour wash in TBST.

For the 2-Mercaptoethanol/SDS (2ME/SDS) wash, we followed the protocol laid out by Gendusa, Scalia et al. (2014). In short, 200mL of 10% w/v SDS was combined with 12.5ml of 0.5M Tris-HCl (adjusted to pH 6.8 at RT) and 67.5mL of dH_2O. 800µl of 2-Mercaptoethanol

was added to this solution and heated to 56°C and incubated for 30 minutes. Sections were then washed in four 15-minute dH_2O and 5 minutes in TBST. Following both elution steps, sections were re-blocked in 5% nonfat milk with 0.05% tween.

Imaging and interpretation

Sections were examined first with the Cy5 channel at 20x to determine if the primary antibody worked (i.e. confirming that there was a significant amount of target in the sample and that the dilution used was sufficient). The DsRed channel was examined for overlapping signal with Cy5. The presence of signal in this channel would indicate that the elution did not remove the antibody complex from the first primary incubation and that AF546 was able to deposit onto the tissue. Last, the GFP channel confirmed that the antigenicity was preserved through the elution step by observing the presence of overlapping signal with Cy5 (Figure 1). Table 2 indicates the intended interpretation of signal combinations from different channels.

Table 2: Interpretation of overlapping signals from each channel in different combinations.

Cy5 (AF647)	**DsRed (AF546)**	**GFP (AF488)**	**Interpretation**
++/+++	-/+	++/+++	Positive elution, preserved antigenicity
++/+++	++/+++	++/+++	Negative elution, unknown effect on antigenicity
++/+++	-/+	-/+	Positive elution, damaged antigenicity
-/+	-/+	-/+	Negative experiment
-/+	-/+	++/+++	Negative experiment

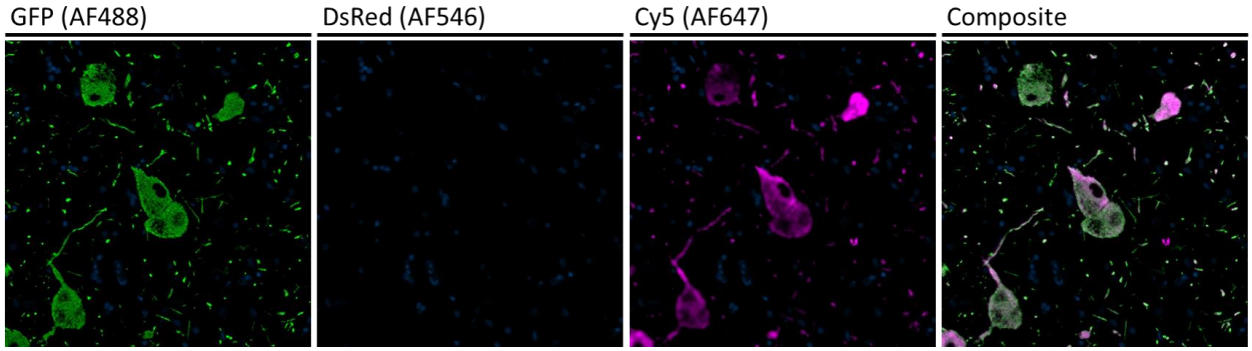

Figure 1: Tyrosine hydroxylase in the locus coeruleus before elution (AF647), after elution (AF546), and after restaining (AF488). This combination of overlapping signals denotes a positive elution and preservation of target antigenicity.

Results

In Table 3, the presence of signal for each antibody/elution/fixation time is qualitatively ranked from "-" (no signal) to "+++" (excellent signal). A "+" is considered to be signal that would fall significantly below the threshold of detection with standard microscopy parameters and thus would be considered to be acceptable elution strength and insufficient antigenicity. There was noticeably less signal in the Cy5 channel than the GFP channel for several antibodies. There were no cases of tissue falling off the section or taking on other frank deformations.

Table 3: Results of elution and preservation of antigenicity. Signal from AF647 denotes successful antibody reactivity in the respective sample. Signal from AF546 denotes a failed elution. Signal from AF488 denotes a successful preservation of antigenicity.

Antibody	Elution	Fixation	Cy5 (AF647)	DsRed (AF546)	GFP (AF488)
CP13	2ME/SDS	Short-fix	+++	-	+++
CP13	Urea/SDS/Glycine	Short-fix	+++	-	+++
T231	2ME/SDS	Short-fix	+++	+	+++
T231	Urea/SDS/Glycine	Short-fix	++	-	+++
GFAP	2ME/SDS	Short-fix	++	-	+++
GFAP	Urea/SDS/Glycine	Short-fix	+++	-	+++
GFAP	2ME/SDS	Long-fix	++	-	++
GFAP	Urea/SDS/Glycine	Long-fix	+	-	++
TMEM119	2ME/SDS	Short-fix	+++	-	+++
TMEM119	Urea/SDS/Glycine	Short-fix	+++	+	+++
TMEM119	2ME/SDS	Long-fix	-	-	-
TMEM119	Urea/SDS/Glycine	Long-fix	-	-	-
PH8	2ME/SDS	Short-fix	++	+	+++
PH8	Urea/SDS/Glycine	Short-fix	+++	-	+++
PH8	2ME/SDS	Long-fix	++	-	+++
PH8	Urea/SDS/Glycine	Long-fix	++	-	++
TH	2ME/SDS	Short-fix	+	-	++
TH	Urea/SDS/Glycine	Short-fix	+	-	++
TH	2ME/SDS	Long-fix	+++	-	+++
TH	Urea/SDS/Glycine	Long-fix	++	-	+++

Discussion

To maximize the use of post-mortem tissue for the study of neurodegenerative diseases, there is an unmet need for reliable strategies for multiplex immunofluorescence. A key step for multiplex immunofluorescence is the elution and removal of antibody complexes. As extreme pH and temperature do not reliably elute antibodies, there is a need for alternative elution strategies that disrupt the antibody-antigen interaction or the structure of the antibody itself (Wang, Singh et al. 2007, Argentieri, Pilla et al. 2013, Gendusa, Scalia et al. 2014).

In this study, we test two elution techniques that have previously been successful with antibodies of different target types and in other tissue types. Across the board, both techniques appeared to sufficiently elute all antibodies tested and the antigenicity was preserved, aligning with previous studies (Gendusa, Scalia et al. 2014). 2ME, a strong reducing agent, acts selectively on disulfide bonds. This allows it to act selectively on antibodies (Capel, Gerlag et al. 1980). Interestingly, the use of 2ME/SDS does not appear to significantly affect the antigenicity of targets enriched for disulfide bonds and only significantly effects the target antibodies, making it an ideal reagent for elution (Gendusa, Scalia et al. 2014).

For several antibodies, there was noticeably less signal in the Cy5 channel than the GFP channel. This could be explained by differences in the strength of the associated fluorophores, but as

strong reducing agents, it should be considered that the reagents used in these elution strategies may be acting on the Alexa Fluor dyes. This should be considered when determining the order of antibodies used in an experiment.

Given the lack of differences between the Urea/SDS/Glycine and the 2ME/SDS elution strategies here, an important consideration is the preservation of tissue integrity long-term. Here, only one round of staining was used, but future studies are needed to assess how tissue integrity might be affected by the techniques after multiple rounds, particularly given the high temperatures involved in the Urea/SDS/Glycine strategy. In fact, the parameter that appears to have most significantly affected the results of this experiment is the fixation time of the samples, illustrating the need for optimization of antibodies and elution for the whole spectrum of sample types to be included in a study.

Taking this study together with previous literature, there is evidence that antibodies can be reliably removed from tissue sections using protocols derived from techniques such as western blot. It is clear that attention must be paid in every experiment to ensure and confirm successful elution by including appropriate controls into the experimental design. Among the parameters considered when optimizing antibodies, elution steps can be selected and optimized to match the unique needs of the antibody, tissue sample, and project.

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