Colocalizing Telomeres With PML or γH2AX Foci by IF-FISH in Mouse Brain Neurons

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Abstract

Telomere length maintenance is strongly linked to cellular aging, as telomeres progressively shorten with each cell division. This phenomenon is well-documented in mitotic, or dividing, cells. However, neurons are post-mitotic and do not undergo mitosis, meaning they lack the classical mechanisms through which telomere shortening occurs. Despite this, neurons retain telomeres that protect chromosomal ends. The role of telomeres in neurons has gained interest, particularly in the context of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), where aging is a major risk factor. This has sparked interest in investigating telomere maintenance mechanisms in post-mitotic neurons. Nevertheless, most existing telomere analysis techniques were developed for and optimized using mitotic cells, posing challenges for studying telomeres in non-dividing neuronal cells. Thus, this protocol adapts an already established technique, the combined immunofluorescence and telomere fluorescent in situ hybridization (IF-FISH) on mitotic cells to study the processes occurring at telomeres in cortical neurons of the mouse ALS transgenic model, TDP-43 rNLS. Specifically, it determines the occurrence of DNA damage and the alternative lengthening of telomeres (ALT) mechanism through simultaneous labeling of the DNA damage marker, γH2AX, or the ALT marker, promyelocytic leukemia (PML) protein, together with telomeres. Therefore, the protocol enables the visualization of DNA damage (γH2AX) or the ALT marker (PML) concurrently with telomeres. This technique can be successfully applied to brain tissue and enables the investigation of telomeres specifically in cortical neurons, rather than in bulk tissue, offering a significant advantage over Southern blot or qPCR-based techniques.
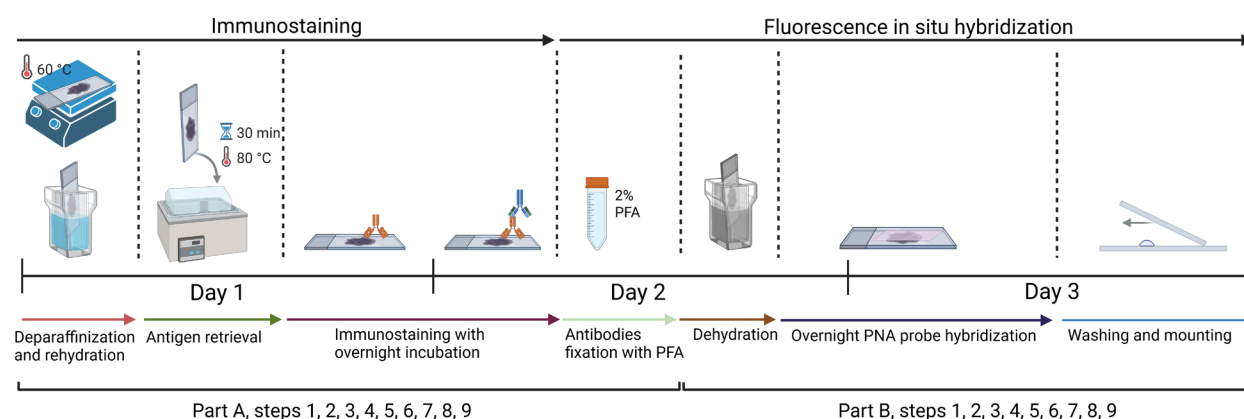
Key features

- This protocol enables the labeling of telomeres in mouse brain tissue prepared from paraffin-embedded brain sections.
- This method facilitates concurrent labeling of proteins that are co-localized at telomere sites.

Keywords: IF-FISH, Telomere, Telomere maintenance, DNA damage, PML, Mouse neurons, Neurodegeneration, ALS

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Graphical overview

Immunostaining | Fluorescence in situ hybridization

60 °C | 30 min / 80 °C | 2% PFA

Day 1 | Day 2 | Day 3

Deparaffinization and rehydration | Antigen retrieval | Immunostaining with overnight incubation | Antibodies fixation with PFA | Dehydration | Overnight PNA probe hybridization | Washing and mounting

Part A, steps 1, 2, 3, 4, 5, 6, 7, 8, 9 | Part B, steps 1, 2, 3, 4, 5, 6, 7, 8, 9

Approximate timeline for conducting immunofluorescence and telomere fluorescent in situ hybridization (IF-FISH)

Background

This protocol has the potential to advance the study of telomere length maintenance mechanisms, particularly in neurons of transgenic mouse models of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS). As post-mitotic cells, neurons do not undergo cell division [1]; therefore, telomere homeostasis is likely regulated differently in these cells compared to mitotic cells, in which telomeres progressively shorten with each round of replication [2]. Telomere length maintenance represents an emerging and compelling area of research, especially in the context of pathological conditions such as ALS that affect the nervous system [3,4].

Established methods for studying telomeres include Southern blot analysis, which enables measurement of terminal restriction fragment (TRF) length [5], and quantitative PCR (qPCR)-based techniques [6], which estimate relative telomere length by comparing telomeric repeat copy number to that of a single-copy gene. While these methods are useful, they often lack cell-type specificity and are not well-suited for analyzing telomere dynamics in heterogeneous tissues such as the brain. ALS affects specific neuronal subtypes in the brain and spinal cord [7]; therefore, a method that enables telomere analysis within the desired cell type is particularly valuable for improving the understanding of the disease mechanism. Moreover, these techniques do not allow for the investigation of molecular events at telomeres, e.g., telomere dysfunction-induced foci (TIFs), and the occurrence of DNA damage or the recruitment of telomere-associated proteins.

In contrast, the described technique for immunofluorescence combined with fluorescence in situ hybridization (IF-FISH) enables the visualization and analysis of telomeric or telomere-associated proteins at telomeric sites, such as TRF2 or Rif1, thereby providing deeper insights into telomere-related molecular processes. This method is particularly valuable in studies requiring cell-type specificity and spatial resolution, making it especially suitable for complex tissues like the brain. While IF-FISH is less suitable for precise telomere length quantification compared to methods such as Southern blot or qPCR, it nonetheless allows for the evaluation of relative telomere length based on fluorescence intensity. Thus, although this approach does not yield exact telomere length measurements, it is still valuable for detecting and comparing telomere length differences between experimental groups. Furthermore, IF-FISH provides spatial information at the single-cell level. When combined with quantitative data from methods such as qPCR or Southern blotting, it enhances the overall interpretation by offering critical context regarding cell-to-cell heterogeneity and the spatial organization of telomeres. This integrative approach enables a more comprehensive and multidimensional analysis of telomere dynamics under various experimental conditions. However, the limitation of the IF-FISH method is its relatively time-consuming protocol, particularly when compared to qPCR-based approaches.

Materials and reagents

Biological materials

1. Mouse brain paraffin sections, 10 μm thick (derived from transgenic bigenic rNLS mouse or monogenic control mouse [8])

Reagents

1. Trisodium citrate·2H$_2$O (Merck, catalog number: 1064480500)
2. Tween-20 (Merck, catalog number: 11332465001)
3. Normal donkey serum (NDS) (Jackson ImmunoResearch, catalog number: 017-000-121)
4. Histone H2AX [p Ser139] antibody (Novus Biologicals, catalog number: NB100-384)
5. Anti-PML antibody, mouse monoclonal (Merck-Millipore, catalog number: P6746-200UL)
6. Tris base (Merck, catalog number: 252859-500G)
7. Sodium chloride (Merck, catalog number: S9625-1KG)
8. Potassium chloride (Merck, catalog number: P3911-500G)
9. Sodium phosphate dibasic (Merck, catalog number: S9763-500G)
10. Potassium phosphate monobasic (Merck, catalog number: P0662-500G)
11. Xylene (histological grade), (Merck, catalog number: 534056-500ML)
12. Alexa Fluor^{TM} 488, anti-mouse (Invitrogen, catalog number: A-21202)
13. Alexa Fluor^{TM} 488, anti-rabbit (Invitrogen, catalog number: A-21206)
14. Paraformaldehyde, 16% (Thermo Fisher Scientific, catalog number: 043368.9M)
15. Texas Red-conjugated C-strand telomere PNA probe (stock solution of 0.3 μL/mL in Mili-Q water) (Panagene, custom fluorescent dye)
16. Blocking reagent (Merck, catalog number: 11096176001)
17. Formamide (Merck, catalog number: F7503-100ML)
18. Hydrochloric acid (Merck, catalog number: 258148-25ML)
19. DAPI (Merck, catalogue number: D9542-1MG)

Solutions

1. 10 mM citrate buffer containing 0.1% Tween-2, pH 6.0 (see Recipes)
2. 1× PBS, pH 7.4 (see Recipes)
3. 1× PBS, pH 7.4 with 0.1% Triton X-100 (see Recipes)
4. 5% NDS (see Recipes)
5. Histone H2AX [p Ser139] antibody 1:200 dilution (see Recipes)
6. Anti-PML antibody 1:200 dilution (see Recipes)
7. 90% ethanol (see Recipes)
8. 70% ethanol (see Recipes)
9. 10 mM Tris Cl, pH 7.5 (see Recipes)
10. 50 mM Tris Cl, pH 7.5 (see Recipes)
11. PNA wash buffer A (70% v/v formamide, 10 mM Tris Cl, pH 7.5) (see Recipes)
12. PNA wash buffer B (50 mM Tris Cl, pH 7.5, 150 mM NaCl, 0.8% v/v Tween 20) (see Recipes)
13. 2% paraformaldehyde in 1× PBS (see Recipes)
14. PNA hybridization solution (see Recipes)
15. PNA probe hybridization working solution (see Recipes)

Recipes

1. 10 mM citrate buffer containing 0.1% Tween-20, pH 6.0

Reagent	Final concentration	Quantity or volume
Trisodium citrate·2H$_2$O (MW = 294.10 g/mol)	10 mM	2.94 g
Milli-Q water	-	To 1,000 mL total volume
Tween-20	0.1% v/v	1.0 mL
Total		1,000 mL

If necessary, adjust to pH 6.0 with HCl.

2. 1× PBS, pH 7.4

Reagent	Final concentration	Quantity or volume
Sodium chloride (MW = 58.44 g/mol)	137 mM	8 g
Potassium chloride (MW = 74.55 g/mol)	2.7 mM	0.2 g
Sodium phosphate dibasic (MW = 141.96 g/mol)	10 mM	1.44 g
Potassium phosphate monobasic (MW = 136.09 g/mol)	1.8 mM	0.245 g
Milli-Q water	-	To 1,000 mL total volume
Total volume		1,000 mL

Adjust to pH 7.4 with HCl.

3. 1× PBS, pH 7.4 with 0.1% Triton X-100

Reagent	Final concentration	Quantity or volume
Sodium chloride (MW = 58.44 g/mol)	137 mM	8 g
Potassium chloride (MW = 74.55 g/mol)	2.7 mM	0.2 g
Sodium phosphate dibasic (MW = 141.96 g/mol)	10 mM	1.44 g
Potassium phosphate monobasic (MW = 136.09 g/mol)	1.8 mM	0.245 g
Milli-Q water	-	To 1,000 mL total volume
Triton X-100	0.1% v/v	1.0 mL
Total		1,000 mL

Adjust to pH = 7.4 with HCl.

4. 5% NDS

Reagent	Final concentration	Quantity or volume
Normal donkey serum	5%	0.5 mL
1× PBS with 0.1% Triton X-100	-	9.5 mL
Total		10 mL

5. Histone H2AX [p Ser139] antibody 1:200 dilution

Reagent	Final concentration	Quantity or volume
5% NDS	5%	199 μL
Histone H2AX [p Ser139] antibody	5 μg/mL	1 μL
Total volume per one slide		200 μL

6. Anti-PML antibody 1:200 dilution

Reagent	Final concentration	Quantity or volume
5% NDS	5%	199 μL
Anti-PML antibody	10–12.5 μg/mL	1 μL
Total volume per one slide		200 μL

7. 90% ethanol

Reagent	Final concentration	Quantity or volume
Absolute ethanol (100%)	90%	225 mL
Milli-Q water	-	25 mL

Total		250 mL

8. 70% ethanol

Reagent	Final concentration	Quantity or volume
Absolute ethanol (100%)	70%	175 mL
Milli-Q water	-	75 mL
Total		250 mL

9. 10 mM Tris Cl, pH 7.5

Reagent	Final concentration	Quantity or volume
Tris base (MW = 121.14 g/mol)	10 mM	1.21 g
Milli-Q water	-	up to 1,000 mL
Total		1,000 mL

Adjust to pH 7.5 with HCl.

10. 50 mM Tris Cl, pH 7.5

Reagent	Final concentration	Quantity or volume
Tris base (MW = 121.14 g/mol)	10 mM	6.06 g
Milli-Q water	-	up to 1,000 ml
Total		1,000 mL

Adjust to pH 7.5 with HCl.

11. PNA wash buffer A (70% v/v formamide, 10 mM Tris Cl, pH 7.5)

Reagent	Final concentration	Quantity or volume
10 mM Tris Cl, pH 7.5	-	150 mL
Formamide	70%	350 mL
Total		500 mL

12. PNA wash buffer B (50 mM Tris Cl, pH 7.5, 150 mM NaCl, 0.8% v/v Tween 20)

Reagent	Final concentration	Quantity or volume
50 mM Tris Cl, pH 7.5	50 mM	490 mL
Sodium chloride (58.44 g/mol)	150 mM	4.39 g
Tween-20	0.8% v/v	4 mL
50 mM Tris Cl, pH 7.5	50 mM	To 500 mL total volume
Total		500 mL

13. 2% paraformaldehyde in 1× PBS

Reagent	Final concentration	Quantity or volume
16% paraformaldehyde	2%	1.25 mL
1× PBS, pH 7.4		8.75 mL
Total		10 mL

14. PNA hybridization solution

Reagent	Final concentration	Quantity or volume
10 mM Tris Cl, pH 7.5	-	2.975 mL
Formamide	70%	7 mL
Blocking reagent	0.25%	25 μL
Total		10 mL

15. PNA probe hybridization working solution

Reagent	Final concentration	Quantity or volume
PNA hybridization solution	-	999 μL
0.3 μg/mL C-strand telomere PNA probe	0.3 ng/mL	1 μL

Total	1,000 μL

Laboratory supplies

1. Parafilm® M sealing film (Merck, catalog number: HS234526B-1EA)
2. Cover glasses (Merck, catalog number: C9802)
3. Coplin jars, e.g., BRAND® plastic staining trough, Hellendahl pattern (Merck, catalog number: BR474400)
4. Dark humidity chamber for slides, e.g., StainTray slide staining system (Merck, catalog number: Z670146-1EA)
5. ProLong™ Gold Antifade Mountant (ThermoFisher scientific, catalog number: P36930)
6. Deionized water

Equipment

1. Hot plate, e.g., benchmark hotplate (Merck, catalog number: Z742547)
2. Drying oven, e.g., binder multifunction drying oven FED series (Merck, catalog number: Z604011)
3. Water bath with heating, e.g., MyBath™ mini water bath (Merck, catalog number: Z741427)

Procedure

A. Immunofluorescence protocol on paraffin-embedded brain sections

Note: Turn on the water bath and set it to heat to 80 °C. Allow the bath to reach and stabilize at that temperature before placing slides inside later in the protocol.

1. Remove paraffin and rehydrate sections
a. Heat slides for 45 min at 60 °C on a hot plate; alternatively, a drying oven can be used.
Note: The following steps are performed at room temperature.
b. Immerse slides directly into pure xylene for 5 min, then repeat for a second 5 min.
c. Then, rehydrate slides with:
100% ethanol (2×, each 3 min)
90% ethanol (3 min)
70% ethanol (3 min)
d. Rinse slides in distilled water for 3 min.

2. Perform antigen retrieval
a. Incubate the slides in 10 mM citrate buffer containing 0.1% Tween-20, pH 6.0, at 80 °C in a water bath for 30 min, then leave them at RT to cool to room temperature.
Note: It is recommended to prepare the buffer fresh for each use; alternatively, it can be stored at 4 °C for up to two weeks.
b. Rinse in 1× PBS.

3. Block nonspecific binding
Note: Place the slides directly into a humidified, dark chamber. Although the dark chamber is not strictly required at this stage, starting in it removes the need to switch chambers later in the protocol.
a. Incubate slides for 30 min at room temperature in 200 μL of 5% NDS in 1× PBS 0.1% Triton per slide to reduce background staining.
Note: Optionally, draw a hydrophobic barrier around the tissue using a PAP pen. This step is not strictly required because the reagent drop usually remains stable.

4. Apply primary antibody
a. Dilute primary antibody (anti-γH2AX 1:200, 5 μg/mL or PML 1:200, 10–12.5 μg/mL) in 5% NDS to obtain 200 μL of solution per slide.
b. Incubate overnight at 4 °C in a humidified chamber.

5. Wash slides

a. Wash slides three times in 1× PBS with 0.1% Triton for 5 min. If a standard 5-slide Coplin jar is used for the washing step, 50 mL of buffer is required per wash.

6. Apply secondary antibody

a. Dilute Alexa Fluor 488-conjugated secondary antibody (1:200), 200 μL per slide.

b. Apply the solution on the slides and incubate for 2 h at RT in a dark, humidified chamber.

7. Wash slides

a. Wash slides two times in 1× PBS with 0.1% Triton for 5 min and once with 1× PBS for 5 min. If a standard 5-slide Coplin jar is used for the washing step, 50 mL of buffer is required per wash.

8. Fix antibody complexes

a. Fix slides in 2% paraformaldehyde for 10 min at room temperature, applying 200 μL of solution to cover the tissue.

9. Final wash

a. Rinse slides three times in deionized water in a Coplin jar. If a standard 5-slide Coplin jar is used for the washing step, 50 mL of buffer is required per wash.

B. PNA probe hybridization (fluorescent in situ hybridization)

Note: Place the hot plate in a dark or dimly lit room. Turn on the hot plate and set it to heat to 80 °C.

1. Dehydrate the slides through a graded ethanol series

Note: The following steps are performed at room temperature.

a. 70% for 5 min

b. 90% for 5 min

c. 100% for 5 min

d. Air-dry the slides, taking care not to over-dry them.

2. Apply 20–50 μL of the PNA probe hybridization working solution (0.3 ng/mL) and cover with a coverslip.

Note: The easiest way to do this is to apply the solution onto the coverslip first (placed on the bench) and then attach it to the microscope slide. Please see Figure 1.

Figure 1. Demonstration of PNA probe application on a glass slide

3. Place the slides on a pre-heated surface at 80 °C for 5 min, keeping them in the dark. Then, immediately transfer them to a humidified chamber in the dark and allow hybridization to proceed overnight at room temperature.

4. Remove the coverslip.

Note: Gently tap the edge of the disposable container with the hand holding the slide. The coverslip usually detaches easily. Please see Figure 2.

Figure 2. Demonstration of the removal of a coverslip

5. Wash the slides in PNA wash buffer A at room temperature, with shaking, 2 times for 10 min each.

Critical: Always prepare PNA wash buffer A fresh, immediately before each use. It is recommended to perform the wash in a Coplin jar, such as a standard 5-slide Coplin jar, which requires 50 mL of PNA wash buffer A per wash.

6. Wash the slides in PNA wash buffer B at room temperature, with shaking, 5 times for 5 min each.

Critical: Always prepare PNA wash buffer B fresh, immediately before each use. It is recommended to perform the wash in a Coplin jar, such as a standard 5-slide Coplin jar, which requires 50 mL of PNA wash buffer B per wash.

Note: Optionally stain with DAPI during the fourth wash if needed.

7. Rinse the slides 3 times in deionized water.

Critical: A proper rinse with deionized water helps shield the tissue from salt-induced damage during the subsequent dehydration step.

8. Again, dehydrate the slides through a graded ethanol series:

70% for 5 min

90% for 5 min

100% for 5 min.

9. Air-dry the slides and mount them in mounting medium. Figure 3 shows an example of the IF-FISH to detect PML and telomere in the cortical neurons of the mouse brain. The images were acquired using a Zeiss LSM 880 confocal microscope equipped with a Plan-Apochromat 63×/1.4 Oil DIC M27 objective.
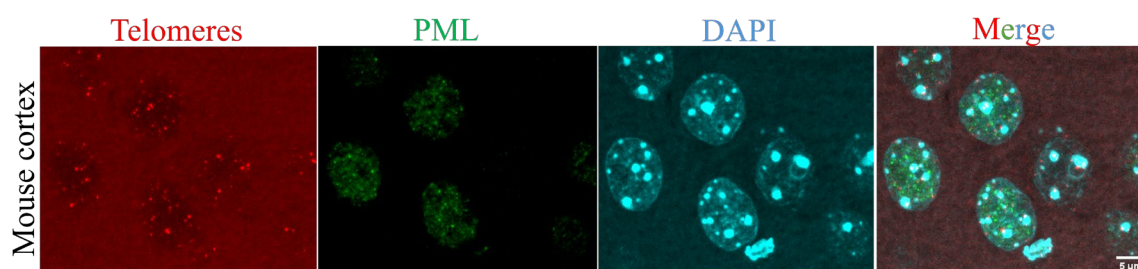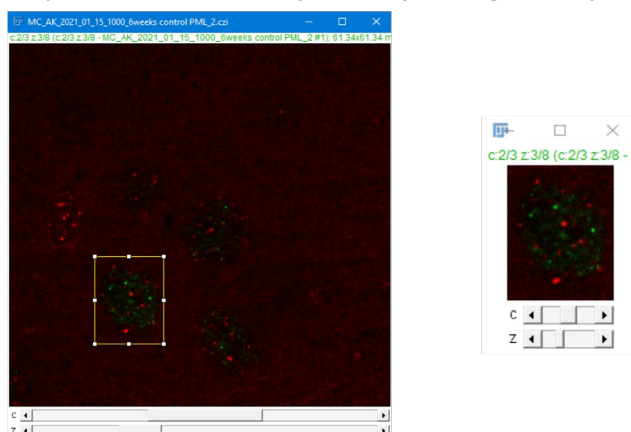

Figure 3. Example of immunofluorescence combined with fluorescence in situ hybridization (IF-FISH) performed on mouse brain tissue. Telomeres (red, by FISH), PML (green, by immunostaining), and nuclei (cyan, by DAPI). Scale bar: 5 μm.
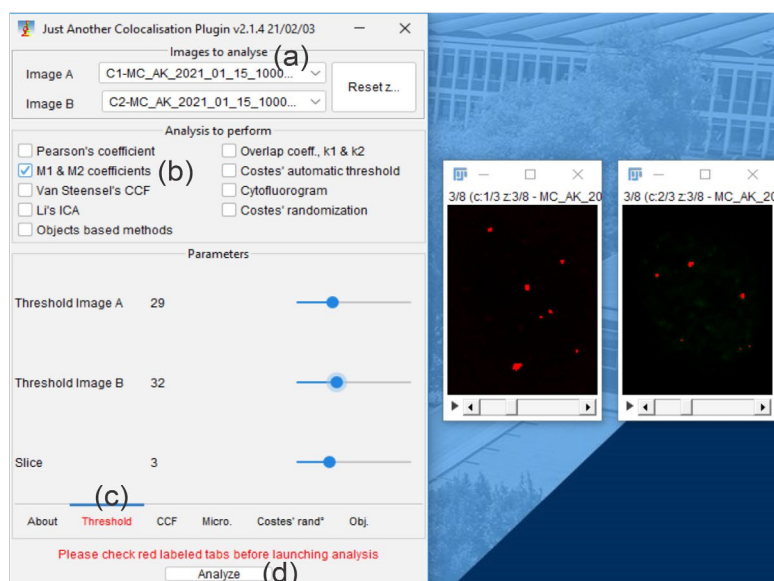
Data analysis

The data analysis was performed as described in the original article [9]. Colocalization between PML bodies or γH2AX foci and telomeres was assessed using Mander's coefficient via the JaCoP plugin [10] in Fiji (ImageJ) software [11]. Single optical slices from confocal z-stacks were used for the analysis. The individual cells were cropped from merged images containing the two relevant fluorescence channels (PML or γH2AX and telomeres). These were then split into separate channels, and after adjusting thresholds, Mander's coefficient was calculated to quantify colocalization. Figure 4 illustrates an example of the workflow.

1. Crop the area for the analysis; In FijiJ, Image -> Crop

2. Split channels; In FijiJ, Image -> Colour -> Split channels
3. Load the images (a) and choose the desired analysis to perform (b).

4. Adjust the threshold (c) if necessary, and analyze (d).

Figure 4. Colocalization analysis using the JaCoP plugin in Fiji (ImageJ) software

Validation of protocol

This protocol has been used and validated in the following research article:
- Konopka et al [9]. Pathological forms of TDP-43 in amyotrophic lateral sclerosis (ALS) promote aberrant telomere elongation. *Biochim Biophys Acta Mol Basis Dis* (Figure 4A, B; Figure 5A, B).

General notes and troubleshooting

General notes

The protocol was optimized and validated using mouse tissue. However, applying it to human tissue did not yield satisfactory staining quality. A potential reason for this could be the quality of preserved brain section morphology. In this case, a neurodegenerative disease (ALS) was investigated, and the specimens were collected at the terminal stage, when extensive neurodegeneration is typically observed.

To strengthen the rigor of the study, the inclusion of the following negative controls is proposed: (1) omission of the primary antibody for immunostaining, (2) omission of the PNA probe, and/or (3) use of a scrambled PNA probe with a meaningless sequence.

Troubleshooting

Problem 1: Weak immunofluorescence signal.
Possible cause: The formamide treatment during PNA probe hybridization.
Solution: It is possible to perform staining after hybridization. If staining is to be performed after PNA hybridization, then the steps involving incubation with the primary and secondary antibodies should be carried out only after the wash step using PNA wash buffer B at step B6. Please note that antigen retrieval should be performed in the original order.

Acknowledgments

Competing interests

The authors declare no conflicts of interest.

Ethical considerations

All experimental protocols, including animal care and handling, were conducted under the approval of the Internal Biosafety Committee of Macquarie University (NLRD 5974–52019597412350) and the Animal Ethics Committee of Macquarie University, New South Wales, Australia (ARA 2015/042, ARA 2017/020-5).

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