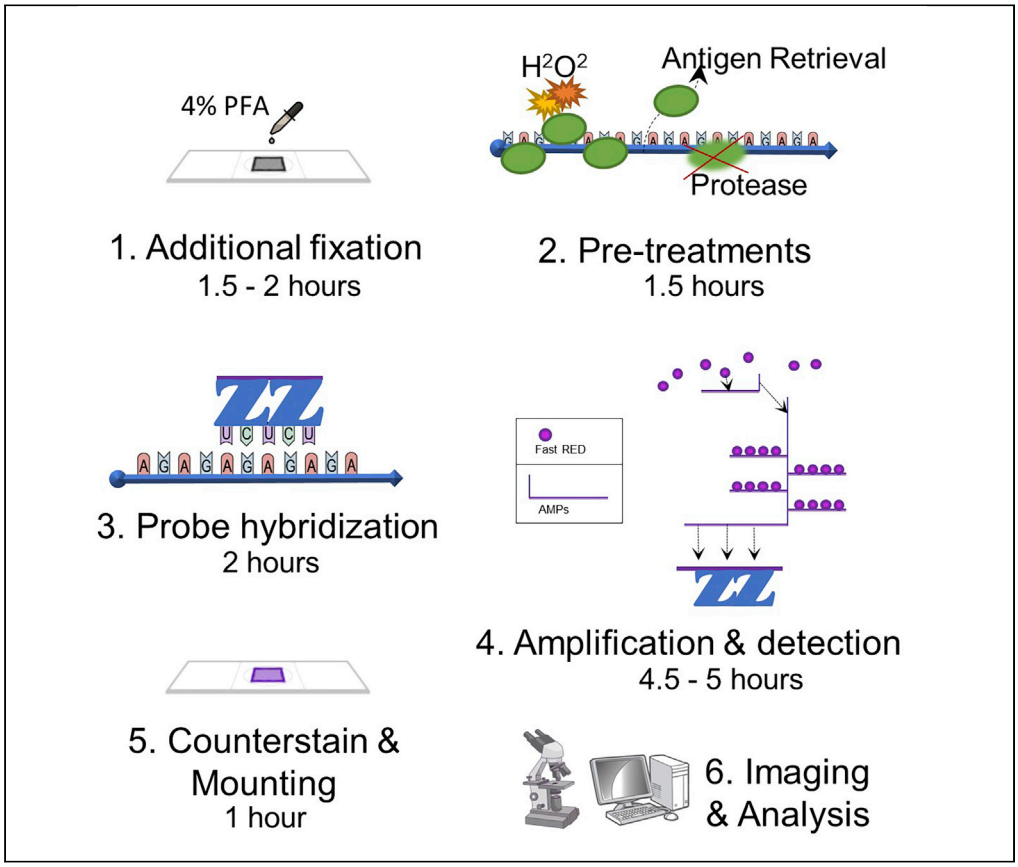
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Analysis of target mRNAs in the fixed-frozen human brain using a modified BaseScope-ISH Assay protocol



We describe a modified BaseScope™ Assay protocol (ACDBio) for RNA *in situ* hybridization on fixed-frozen human brain tissue. The original protocol caused tissue detachment due to harsh tissue pre-treatment. We therefore optimized it to improve tissue stability while providing high stain quality in fragile post-mortem tissue from aged donors with advanced neurodegeneration. The main changes include two additional fixation steps and modifications to the pre-treatment protocol. We also describe tissue imaging and stain quantification using the open-source QuPath software.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

M. Carla Carisì, Owain W. Howell, Alwena H. Morgan, Jeffrey S. Davies

mariacarla.carisi@gmail.com (M.C.C.)
jeff.s.davies@swansea.ac.uk (J.S.D.)

Highlights

Modified BaseScope™ v2 Assay protocol (ACDBio) to increase tissue stability and quality

Uses fixed-frozen post-mortem tissue from donors with advanced neurodegeneration

Two additional fixation steps and changes to the pre-treatment protocol improve outcomes

Steps for tissue imaging and stain quantification using the open-source QuPath software

Carisì et al., STAR Protocols 3, 101896
December 16, 2022
https://doi.org/10.1016/j.xpro.2022.101896

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Analysis of target mRNAs in the fixed-frozen human brain using a modified BaseScope-ISH Assay protocol

M. Carla Carisì,[1,2,*] Owain W. Howell,[1] Alwena H. Morgan,[1] and Jeffrey S. Davies[1,3,*]

[1]Institute of Life Science, Swansea University Medical School, Swansea University, Swansea, Wales SA2 8PP, UK

[2]Technical contact

[3]Lead contact

*Correspondence: mariacarla.carisi@gmail.com (M.C.C.), jeff.s.davies@swansea.ac.uk (J.S.D.)
https://doi.org/10.1016/j.xpro.2022.101896

SUMMARY

We describe a modified BaseScope™ Assay protocol (ACDBio) for RNA *in situ* hybridization on fixed-frozen human brain tissue. The original protocol caused tissue detachment due to harsh tissue pre-treatment. We therefore optimized it to improve tissue stability while providing high stain quality in fragile post-mortem tissue from aged donors with advanced neurodegeneration. The main changes include two additional fixation steps and modifications to the pre-treatment protocol. We also describe tissue imaging and stain quantification using the open-source QuPath software.

For complete details on the use and execution of this protocol, please refer to Hornsby et al. (2020).

BEFORE YOU BEGIN

We recently described the use of post-mortem fixed frozen brain tissue from healthy donors, Parkinson's Disease (PD) patients, and PD patients with dementia (PDD) (Hornsby et al., 2020), using a modified BaseScope™ v2 Assay protocol (ACDBio) (Wang et al., 2012). The protocol below describes the specific steps for using 5–7 μm thick sections of human hippocampal tissue, fixed quickly post-mortem (post-mortem interval $\leq$29 h) for a minimum of 1 week in cold 4% PFA, and stored at −80°C for several years. The protocol includes a 15 min incubation step with antigen retrieval and a 30 min pre-treatment with protease. It can be adapted for use on any fragile on-slide tissue, with minor modifications. More specifically, thicker sections may require harsher treatments, such as longer target retrieval and/or the application of harsher proteases, while sections with an overly strong background staining may need milder treatments (troubleshooting).

⚠ CRITICAL: Age, disease stage, ante-mortem and post-mortem processes, section thickness, and other intrinsic factors can highly influence the outcome of the experiment, therefore we recommend optimizing the protocol for the specific tissue used (Figure 1). Some guidelines for optimisation can be found in the troubleshooting section. For instance, thicker tissues may need longer antigen retrieval, or harsher protease treatment, while aged tissue may be more fragile, hence a milder pre-treatment protocol may be required. When working with aged/damaged tissue, selecting thicker sections - when possible - may help reducing tissue fragility. Lastly, we highly recommend optimising the protocol using both positive (high expression) and negative (negligible expression) housekeeping probes on a small number of cases that represent the samples under investigation. It is recommended to select positive housekeeping probes with similar expression levels and patterns (if known) to the gene of interest. In our protocol, we used the peptidyl-propyl isomerase B (PPIB, accession NG_012979.1) gene as a positive control (Figures 2A and 2B), and the

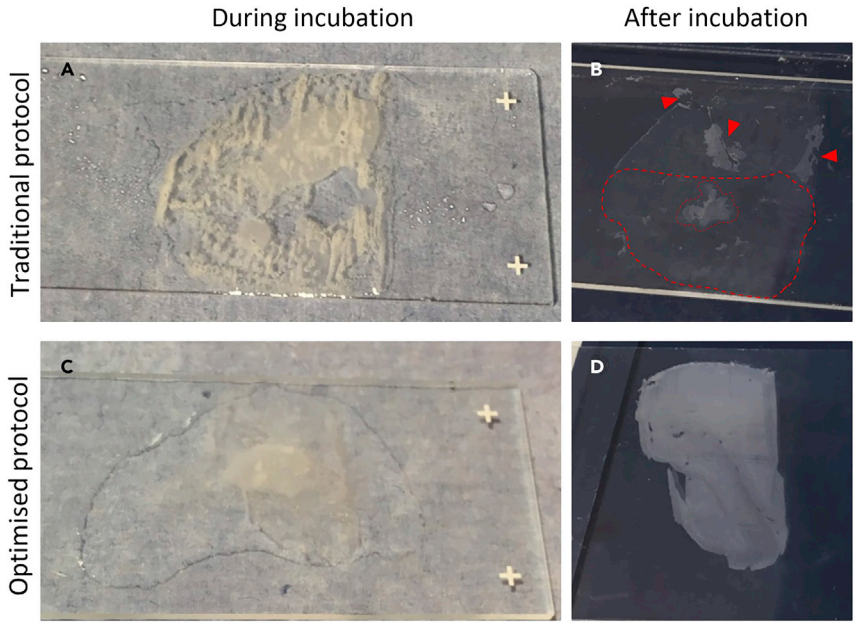


Figure 1. H_2O_2 incubation on fixed frozen brain tissue

(A–D) Comparison of the traditional protocol (A and B), and our optimized protocol (C and D) during (left) and after (right) incubation with H_2O_2 (step 6), one of the most delicate phases of the protocol. Traditional protocol causes extensive tissue loss (B, circled in red), and tissue damage (B, red arrowheads). The extra fixation and baking steps optimized in this protocol improved tissue adherence to the glass, preventing tissue detachment (C and D).

bacterial 4-hydroxy-tetrahydrodipicolinate reductase (DapB, accession EF191515) gene as a negative control (Figure 2C).

⚠ CRITICAL: Use gloves/PPIs and clean instrument thoroughly before starting, using ethanol 70% or RNase-Zap, to minimize cross-contamination. It is recommended to use dedicated bench area and equipment.

Important: It is strongly recommended to collect the tissue on coated and electrostatically (positively) charged glass slides, to prevent the detachment of the tissue sections.

Technical information

The whole procedure can be completed in 11–12 h, conveniently divided across two or more days, via optional pause points. All pause points have been tested and they do not significantly affect the outcome. However, for reproducibility we strongly recommend determining in advance which of these are the most appropriate to one's experimental procedure and use it consistently. In all our experiments, we perform steps 1–18 on day one (5.5–6 h), incubating the slides for 18–24 h with 5×SSC buffer (optional pause point 4), prior to performing step 19 to the end, on day 2 (5.5–6 h).

⚠ CRITICAL: Perform pre-treatments and long incubations horizontally on a flat surface, as a gradient may facilitate tissue detachment from the glass. Short and gentle washes can be performed either horizontally or vertically, in classical IHC racks/glass jars with little risk of detachment.

Important: Each reagent is dispensed in drops, according to the size of the tissue. Determine in advance the number of drops required to submerge the whole tissue (see Table 1 for a reference). Use the same number of drops in each step across all tissues.

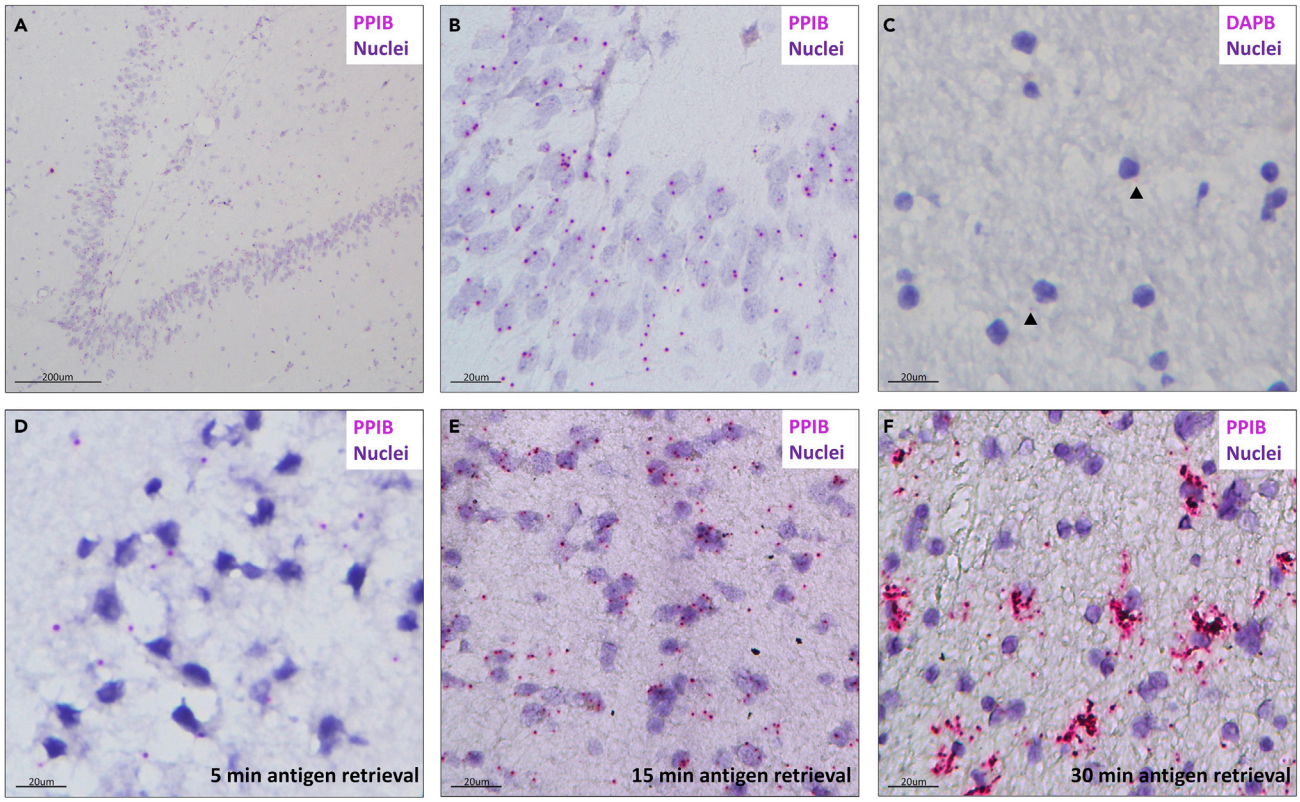


Figure 2. Expected outcomes

(A–C) Human hippocampus expression of the positive housekeeping PPIB probe at 100× magnification (A) and 400× magnification (B), compared to the expression of the negative housekeeping DapB at 400× (C).

(D–F) The bottom line (400×) shows how variations of the antigen retrieval incubation time may influence the expected outcome: 5 min (D), 15 min (E) and 30 min (F).

Note: The present protocol was originally designed on a previous version of the BaseScope™ Assay kit (now discontinued), and later adapted to the newer BaseScope™ v2 Assay kit, with no noticeable differences. Please refer to Figure 3 to compare the differences between the two versions of the assay.

Abbreviations

PD, Parkinson's Disease; PDD, PD with dementia; PPIB, peptidyl-propyl isomerase B; DapB, 4-hydroxy-tetrahydrodipicolinate reductase; SSC, saline-sodium citrate; IHC, immuno-histochemistry; NBF, neutral buffered formalin; PBS, phosphate-buffered saline; PFA, paraformaldehyde; AMP, amplification reagents; ROI, region of interest; GOI, gene of interest; pmi, post-mortem interval; FFPE, formalin-fixed paraffin-embedded.

Table 1. Number of drops and volume of Fast RED[a] mix

Approx. size of the tissue	Drops per section	Recommended volume	Volume of fast red A	Volume of fast red B
1 cm × 1 cm	2	60–70 µL	60 µL	1 µL
1,5 cm × 1,5 cm	3	90–100 µL	90 µL	1,5 µL
2 cm × 2 cm	4	120–130 µL	120 µL	2 µL
2 cm × 2.5 cm	5	150–160 µL	150 µL	2,5 µL
2 cm × 3 cm	6	180–190 µL	180 µL	3 µL

[a]Step 35.

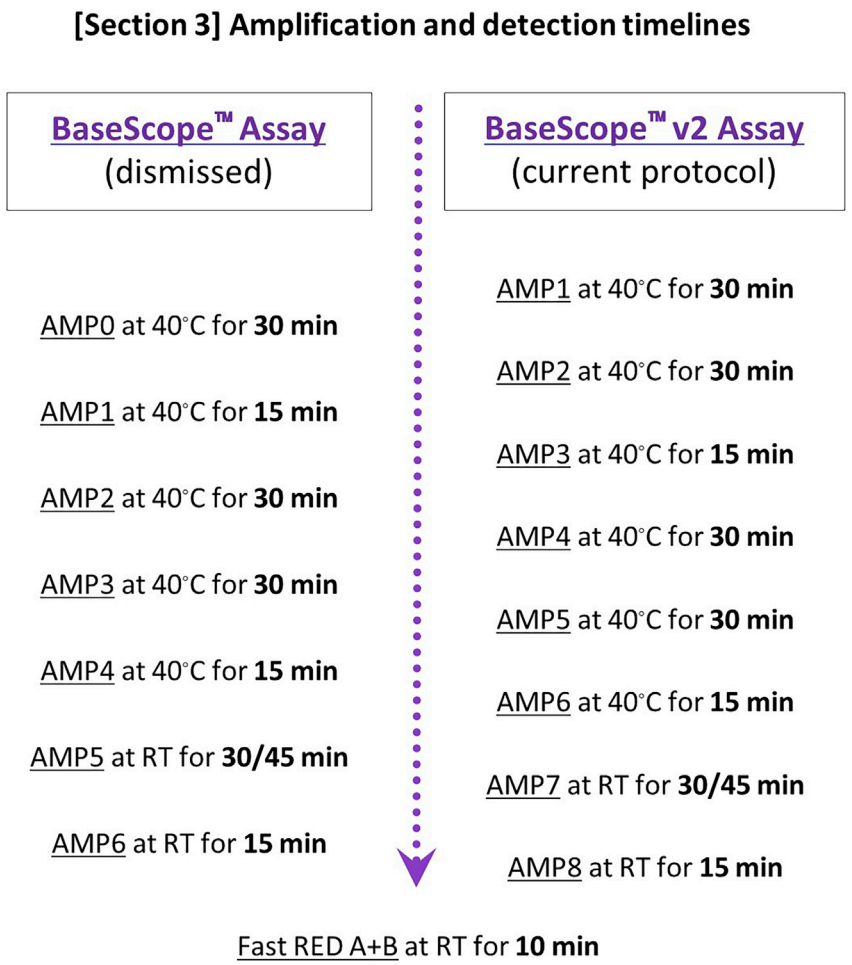


Figure 3. Amplification and detection timelines

In a previous version of the BaseScope™ Assay kit, the amplification steps were called AMP 0–6, for a total of 7 steps. The current BaseScope™ v2 Assay kit has 8 amplification steps, from AMP 1–8.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human hippocampus from aged healthy subjects, PD and PDD patients	UK PD Society Tissue Bank at Imperial College, London	N/A
Chemicals		
10% NBF **Harmful:** skin irritation, organ damage, suspected cancerogenic. Handle under a fume hood, using the appropriate PPI.	Sigma-Aldrich	HT501128
Sodium chloride (powder)	Sigma-Aldrich	S9888
Sodium citrate dihydrate (powder)	Sigma-Aldrich	W302600
Hematoxylin	Vector Labs	H-3401
Critical commercial assays		
BaseScope™ Pretreatment kit	ACDBio	https://acdbio.com/universal-pretreatment

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
BaseScope™ Assay kit	ACDBio	https://acdbio.com/basescope%C2%A0red-assay
RNAscope® Wash Buffer Reagents	ACDBio	310091
Deposited data		
Raw and analyzed data	Hornsby et al. (2020)	N/A
Oligonucleotides		
Human PPIB probe	ACDBio	Contact supplier
Bacterial DapB probe	ACDBio	Contact supplier
Probe of interest	ACDBio	Contact supplier
Software and algorithms		
QuPath	University of Edinburgh (Bankhead et al., 2017)	https://qupath.github.io
Other		
SuperFrost® Plus glass slides	Thermo Fisher	630-0950
ImmEdge Hydrophobic Barrier PAP Pen	Vector Labs	H-2000
Food steamer	Braun	FS3000WH
EasyDip™ Slide Staining System by Simport™ Scientific	Fisher Scientific	15340236
Drying and heating oven (from now on referred to as "dry oven")	Binder	9010-0333
HybEZ™ II Hybridization System (from now on referred to as "HybEZ oven")	ACDBio	321710 and 321720
VectaMount® Permanent Mounting Medium	Vector Labs	H-5000

MATERIALS AND EQUIPMENT

Reagent preparation

Important: Use fresh distilled water and graded series of ethanol:water solutions for every run.

1× wash buffer

Timing: 30 min

Prepare 1× wash buffer and store at 20°C–25°C for up to 1 month.

- Warm 1 bottle (60 mL) of the 50× wash buffer at 40°C for 20 min. This can be done by placing the whole bottle in a dry oven or in a water bath, tightly sealed.
- When still warm, add 2.94 L of distilled water and agitate well.

Final volume will be 3 L. If less volume is required, warm a smaller aliquot of the 50× wash buffer and dilute accordingly.

1× target retrieval reagent

Timing: 2 min

- Prepare 100 mL of 1× target retrieval reagent by pipetting 10 mL of the 10× target retrieval reagent to 90 mL distilled water. The reagent can be reused once, on the same day. Otherwise, prepare fresh on the day of the experiment.

20× SSC buffer (optional)

Timing: 10 min

Prepare a stock solution of 20× saline-sodium citrate (SSC) buffer as shown in the following table, and store at 20°C–25°C for up to 1 month.

Ingredients	Quantity	Final concentration
Sodium Chloride	35.0 g	3 M
Sodium Citrate dehydrate	17.64	0.3 M
Water	Up to 160 mL	

- Measure pH: it should be between 7 and 7.8. Adjust if needed.
- Before use, dilute 1:4 with distilled water for a 5× SSC buffer solution and use on the same day.

50% haematoxylin

Timing: 2 min

Prepare 50% Gill's Haematoxylin by diluting it 1:1 in distilled water. It can be prepared in advance and stored at 20°C–25°C for 2–4 weeks. Check before every use for deposits and/or precipitates, and filter if necessary.

STEP-BY-STEP METHOD DETAILS

Section 1 - Extra tissue fixation

Timing: 1.5–2 h

The following steps are needed to stabilize and prevent detachment of tissue from the slide during pre-treatments (i.e., during hydrogen peroxide). This step is particularly required for tissue that is 'weak' as a result of prolonged post-mortem storage, or tissue from aged donors with extensive cell loss derived from an advanced neurodegenerative state.

Note: tissue fixation could be performed a few days in advance, although it is highly recommended to perform it on the same day of the experiment.

⚠ CRITICAL: all steps and washes in section 1 are performed with the glass slides laying on a horizontal surface. Submerging the tissue section and using coplin jars or other vertical containers in these early steps may increase tissue detachment.

1. Place the slides horizontally on a flat surface and let them thaw for 1–2 min on the bench until most of the moisture has evaporated from the tissue.

⚠ CRITICAL: It is essential to not let the tissue over-dry, as this may cause artifacts. The precise timing for the moisture to evaporate will vary depending on the size of the section. Gently tilting the glass slide to visually inspect the tissue from an angle may help to determine the amount of water/ice still present on the tissue surface.

2. Add a thin layer of 10% NBF directly on the tissue and incubate for 60 min at 20°C–25°C.

Note: Use sufficient NBF to cover the entire section, approximately 300 µL per 2 × 2 cm sections.

3. Remove NBF by gently tilting each slide on a paper tissue, then wash by gently adding a generous amount of PBS directly to the glass with a pipette, while still placed on a flat surface. Remove PBS by gently tilting each slide on a paper tissue and repeat washes every 2 min for three times.

Note: Dispose of the paper tissue containing NBF safely as it contains formaldehyde (toxic).

⚠ CRITICAL: Do not let the sections dry during washes.

4. Dehydrate in freshly prepared 70% ethanol for 3 min before moving into 100% ethanol for 3 min.

Pause point 1 (optional): Store the slides in 100% ethanol at −20°C for up to 1 week. Prolonged storage may degrade the RNA.5. Bake at 60°C for 30 min.

Pause point 2 (optional): After baking, leave slides at 20°C–25°C for 18–24 h, protected from dust.*Note:* If continuing the protocol on the same day, leave the oven switched on at 60°C after baking.

Section 2 - Tissue pre-treatment and probe incubation

Timing: 3.5 h

Tissue pre-treatment is a variation of the original supplier protocol. Different tissue quality/fixation may require further optimization (troubleshooting).

Before starting section 2:

Switch on dry oven to 60°C.

Turn on the HybEz oven at 40°C. Place humidifying paper in the tray and wet with distilled water. Warm the tray for at least 20 min before use.

Equilibrate protease and BaseScope probes at 20°C–25°C for at least 30 min before use.

Place two heat-resistant coplin jars in the steamer: one containing 100 mL distilled water and one containing 100 mL of 1× target retrieval reagent. Switch on and pre-heat to 90°C–99°C.

Note: Since the protocol is very time sensitive, use jars with a separate removable slide rack, so that all sections are placed in/removed from the liquid at the same time. We suggest the EasyDip™ Slide Staining System (see key resources table).

⚠ CRITICAL: Steamer will be needed during steps 8 and 9. Make sure temperature reaches at least 90°C before submerging the slides. It is very important that the steamer does not boil dry, but it is also recommended to avoid opening the lid of the steamer or adding cold water during the process, as the antigen retrieval reagent is very sensitive to temperature changes. We suggest testing in advance the duration of the water contained in the tank and the temperature of the steamer for the length of the procedure.

6. Arrange the slides horizontally in the plastic rack provided with the HybEz oven. Add the appropriate number of drops of Hydrogen Peroxide (H_2O_2) directly on the dry tissue and incubate for 10 min at 20°C–25°C.

Suggestion: H_2O_2 (step 6) and proteases (step 14) do not easily spread and show very high surface tension. To facilitate the distribution of the liquid on the tissue, gently use the side of the tip to spread the drop.

⚠ CRITICAL: do not touch the tissue with the tip.

Alternatives: It is also possible to place a small square of parafilm on the drop to facilitate spreading. However, we noticed a reduction in the final signal from the probe when using parafilm to spread the pre-treatment reagents, suggesting a reduction in the efficacy of the reagents.

7. Place the rack in distilled water and move it gently 3–5 times. Repeat once, replacing with fresh water.

⚠ CRITICAL: Do not let the slides dry during washes.

8. Move all the slides into a vertical rack, then place it quickly in the coplin jar with distilled water at 90°C (inside the steamer) for 10 s to acclimatize.
9. Quickly move slides into the coplin jar with 1× target retrieval reagent at 90°C and keep covered for 15 min.

⚠ CRITICAL: Move slides in the steamer quickly and keep the steamer's lid open as little as possible, to prevent drastic changes in temperature.

10. Remove slides from the steamer and transfer to a container with distilled water at 20°C–25°C for 10 s to acclimatize.
11. Wash slides in fresh 100% ethanol for 3 min, then repeat once.
12. Dry slides in the dry oven at 60°C for 20 min.

Pause point 3 (optional alternative): Dry for 18–24 h at 20°C–25°C, protected from dust.

13. Using a hydrophobic barrier pen, draw a barrier 2–4 times around each section and allow it to dry completely at 20°C–25°C.

Note: Do not write on the tissue, as the wax will prevent any stain to develop in the marked area.

⚠ CRITICAL: Make sure the tissue is completely dry before proceeding to step 14.

14. Place slides in the plastic rack, then insert the rack into the aluminium tray (rack and tray are supplied with the HybEz oven). Apply the correct number of drops of protease IV directly on the dry tissue and incubate in the HybEz oven at 40°C for 30 min.
15. After the incubation, remove the plastic rack with the slides from the oven tray, and quickly put the empty tray back into the oven to prevent cooling.
16. Wash the slides in distilled water for 2 min and remove excess liquid by gently shaking the tray/slides. Repeat once.

⚠ CRITICAL: Do not let the slides dry during washes.

17. Place the rack back in the warm oven tray. Add the correct number of drops of the appropriate BaseScope probe. Incubate in the HybEz oven for 2 h at 40°C.
18. Wash once with 1× wash buffer for 2 min at 20°C–25°C and gently shake to remove the excess liquid.

Pause point 4 (optional, recommended): Prepare 100 mL 5× SSC buffer by pipetting 25 mL of the 20× solution in 75 mL distilled water. Place the slides in 5× SSC at 20°C–25°C for 18–24 h.

Section 3 - Amplification and detection

Timing: 5.5–6 h

Amplification and detection are the core part of the protocol and have not been modified to ensure reproducibility and high quality.

⚠ CRITICAL: The timing of the steps in this section must be carefully monitored. A small variation may greatly affect the reproducibility of the protocol.

Note: please refer to Figure 3 for comparison of the following steps with the older version of the BaseScope™ Assay.

Before starting section 3:

Place the 8 bottles of the amplification reagents (AMP 1–8) at 20°C–25°C 30 min before use.

Turn on the HybEz oven at 40°C. Place humidifying paper in the tray and wet with distilled water. Warm the tray for at least 20 min before use.

Switch on dry oven to 60°C.

19. Wash with 1× wash buffer for 2 min at 20°C–25°C and gently shake to remove the excess liquid. All washes from now on will be performed in 1× wash buffer until otherwise stated.

Note: If slides were kept in SSC buffer, repeat the wash twice.

⚠ CRITICAL: Do not let the slides dry during washes.

20. Place the rack back in the warm oven tray. Add the correct number of drops of the AMP1 solution. Incubate in the HybEz oven for 30 min at 40°C.
21. Put the empty tray back into the oven. Wash twice for 2 min at 20°C–25°C and remove excess liquid.
22. Place the rack back in the warm oven tray. Add the correct number of drops of the AMP2 solution. Incubate in the HybEz oven for 30 min at 40°C.
23. Put the empty tray back into the oven. Wash twice for 2 min at 20°C–25°C and remove excess liquid.
24. Place the rack back in the warm oven tray. Add the correct number of drops of the AMP3 solution. Incubate in the HybEz oven for 15 min at 40°C.
25. Put the empty tray back into the oven. Wash twice for 2 min at 20°C–25°C and remove excess liquid.
26. Place the rack back in the warm oven tray. Add the correct number of drops of the AMP4 solution. Incubate in the HybEz oven for 30 min at 40°C.
27. Put the empty tray back into the oven. Wash twice for 2 min at 20°C–25°C and remove excess liquid.
28. Place the rack back in the warm oven tray. Add the correct number of drops of the AMP5 solution. Incubate in the HybEz oven for 30 min at 40°C.
29. Put the empty tray back into the oven. Wash twice for 2 min at 20°C–25°C and remove excess liquid.
30. Place the rack back in the warm oven tray. Add the correct number of drops of the AMP6 solution. Incubate in the HybEz oven for 15 min at 40°C.

Note: Do not insert the empty tray back in the oven for the rest of the procedure.

31. Wash twice for 2 min at 20°C–25°C and remove excess liquid.
32. Add the correct number of drops of the AMP7 solution. Incubate in the HybEz oven for 30 min at 20°C–25°C.

Note: Stain intensity can be improved by increasing AMP7 incubation for up to 45 min.

33. Wash twice for 2 min at 20°C–25°C and remove excess liquid.
34. Add the correct number of drops of the AMP8 solution. Incubate in the HybEz oven for 15 min at 20°C–25°C.
35. During washes, spin down the Fast RED-B tube, then dilute 1:60 to Fast RED-A. See Table 1 for reference; prepare slightly in excess to account for pipetting errors. Mix well and shield from light.

⚠ CRITICAL: Use Fast RED-A-B mix within 5 min of its preparation.

36. Shake the rack to remove excess liquid and place it back inside the tray. Pipette the correspondent volume of the Fast RED mixture, close the lid of the oven tray (supplied) and incubate for 10 min at 20°C–25°C, shielded from light.

⚠ CRITICAL: It is very important that the incubation with Fast RED incubation time is precisely 10 min. Variations in the timing will impact the reproducibility of the experiment.

Note: During optimization, it is possible to check the intensity of the stain using the microscope while it is developing.

⚠ CRITICAL: From this point forward, any contact with alcoholic solutions will completely erase the Fast RED stain.

37. Quickly place sections in a glass jar with tap water. Rinse again with fresh tap water.

Alternatives: Since the Fast RED dye is also fluorescent with red wavelength (555), after this step, the protocol can be multiplexed with immunofluorescence and kept in the dark, as long as the additional protocol does not require ethanol or other alcohol-based solutions (refer to supplier's protocol for more information). In our hands, multiplexing BaseScope with green fluorophore-conjugated antibodies worked on fixed cell monolayers (cell culture), but we did not test this on tissue sections. For information about performing BaseScope in cell cultures, refer to supplier's protocol.

38. Place the slides on a thick paper tissue and use a Pasteur pipette to add some drops of 50% Haematoxylin. Incubate at 20°C–25°C for 1–2 min, until sections turn purple.

Note: Timing may vary according to the thickness/quality of the tissue. It is recommended to test it in advance. Do not overstain.

39. Remove haematoxylin by gently tilting each slide on the paper tissue before submerging the slides in tap water for 2 min. Repeat washes 2–3 times, until the water is clear.
40. Dry slides completely in a 60°C dry oven for 45 min.

Pause point 5 (optional alternative): Leave slides to dry for 18–24 h at 20°C–25°C, protected from dust.

41. When the slides have dried, place them horizontally on a flat surface. Add 1–2 drop of VectaMount directly onto the tissue and place a coverslip. Avoid trapping bubbles and allow to air dry.

Note: Gently drop the mounting media directly onto the tissue from a close distance to prevent tissue damage. If air bubbles form, gently aspirate them using a 10 μL pipette. Perform this step carefully and avoid touching the tissue.

⚠ CRITICAL: To seal the coverslip onto the glass and prevent mounting media leakage, place a few drops of clear nail polish at the edge of each coverslip.

Note: Better quality images are obtained if imaging is performed when mounting media has dried. We recommend imaging the slides the next day for highest quality.

Note: The chromogenic stain from the BaseScope assay is highly stable and can be imaged several months after the end of the procedure. However, if multiplexed with immunofluorescence the signal is less stable; please refer to supplier's protocol for more information.

Section 4 - Image acquisition

Timing: variable

42. The stained tissues can be imaged using any brightfield microscope with scanning function.

Suggestion: In our experiments we used the Axio Scan Z1 (Zeiss) system with a Plan-Apochromat 40×/0.95 KorrM27 objective. The system uses a Hitachi HV-F202SCL camera that acquires 10,000–100,000 different tiles (pictures) and automatically merges them in a single file (Figure 4A), using an 85% JpgXr compression (24bit depth).

EXPECTED OUTCOMES

Each single RNA transcript appears as a distinct red/purple dot of chromogen precipitate. With the positive control probe, PPIB, most of the stain is localized in the perinuclear area (Figure 4B, black arrowheads), whilst sparce signal is present in the white matter (Figure 4B, red arrowheads). Blood vessels and capillaries appear as circular-like empty areas surrounded by dense aggregates of nuclei (Figure 4C, brown arrowheads).

In our fixed-frozen brain tissue, the majority of the cells stained positive for PPIB, yielding 8–10 dots per cell (Figure 4B, black arrowhead). On the contrary, the negative control probe DapB, yields $\leq$1 dot per cell, with mostly cells showing no signal (Figure 2C, black arrowheads).

QUANTIFICATION AND STATISTICAL ANALYSIS

Image analysis was performed using the QuPath opensource software, developed by Edinburgh University (Bankhead et al., 2017). Figure 5 shows the processing of a single tissue section stained for the positive housekeeper gene, PPIB.

Alternatives: A protocol is available from ACDbio that uses other opensource analysis software such as ImageJ (ACDbio Analysis Guidelines, n.d.).

1. Using the selection tool in the QuPath software, select the region of interest (ROI). For this particular experiment, the ROI was the granule cell layer (GCL) of the hippocampus (Figure 5A, circled in red). Once selected, the software automatically calculates the size of the ROI in μm^2 (Figure 5A, bottom left, red arrow).
2. After zooming in on the ROI, use the manual count tool (Figure 5B, green arrow) to identify the single dots throughout the tissue (Figure 5B, circled in yellow); the counted dots appear as a

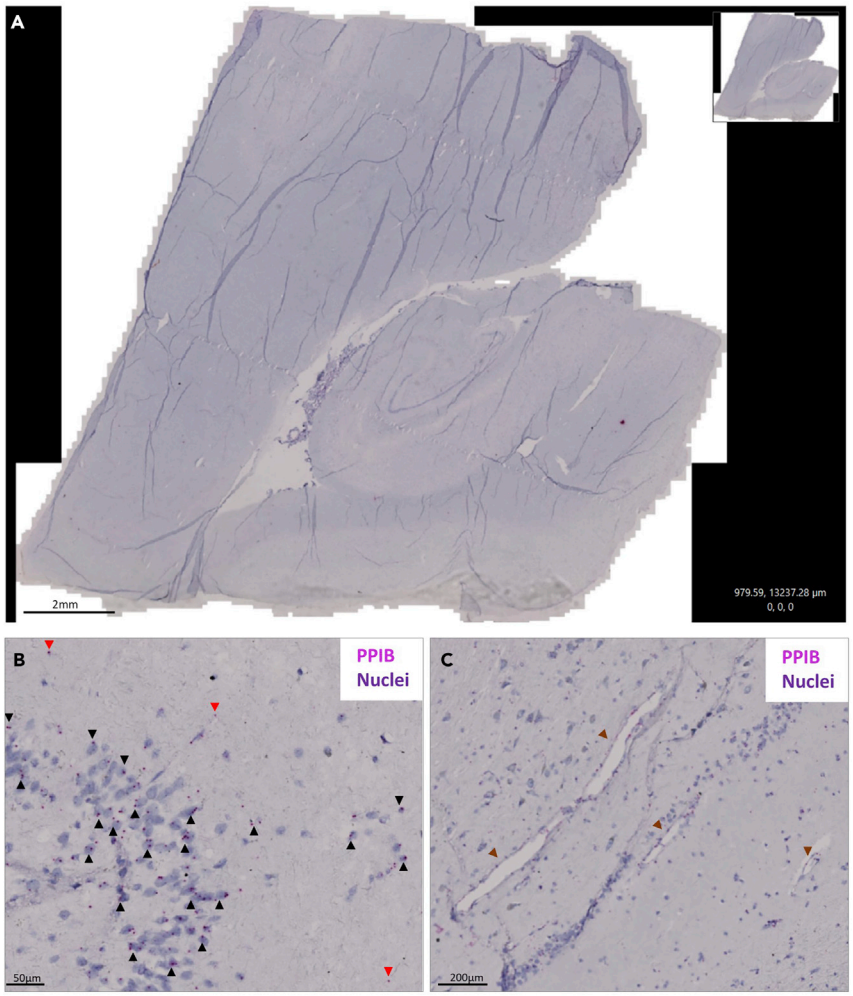


Figure 4. Expected outcomes

(A–C) A scanned hippocampal tissue section in its entirety (A) (approximately 1.7cm × 1.5cm) stained with haematoxylin and PPIB (not visible at this magnification). The image is comprised of "tiles" that have been separately acquired then merged together - giving the tissue a "scattering"-looking border. Each image was acquired at 400× magnification and merged so that it can be magnified on the software without losing resolution. The wrinkles on the tissue are present in all the sections we analysed for this study and are likely caused by prolonged tissue storage at −80°C. When zooming in on unfolded tissue, the tissue appears densely stained with the positive housekeeper PPIB (B), with most of the stain localised in the perinuclear area (black arrowheads), while only sparce signal is visible in the white matter (red arrowheads). Lastly, blood vessels and capillaries (C, brown arrowheads) appear as oval-like shaped holes surrounded by aggregates of nuclei.

mask on top of the picture so that each point can be easily moved or deleted. At the end of this step, the software provides the number of dots selected (Figure 5B, blue arrow). These data can be exported into an Excel spreadsheet for subsequent analysis.

Normalisation 1

In our experiments, some sections presented extensive folds (Figure 5D). These folds resulted in areas that were out of focus following image scanning (Figure 5C). Since these conditions make it difficult to estimate the exact number of dots in the area, folded tissue and out of focus areas were excluded from any ROI. Therefore, the dots that were counted were normalized (divided) by the size of the corresponding ROI (μm^2) (Table 2).

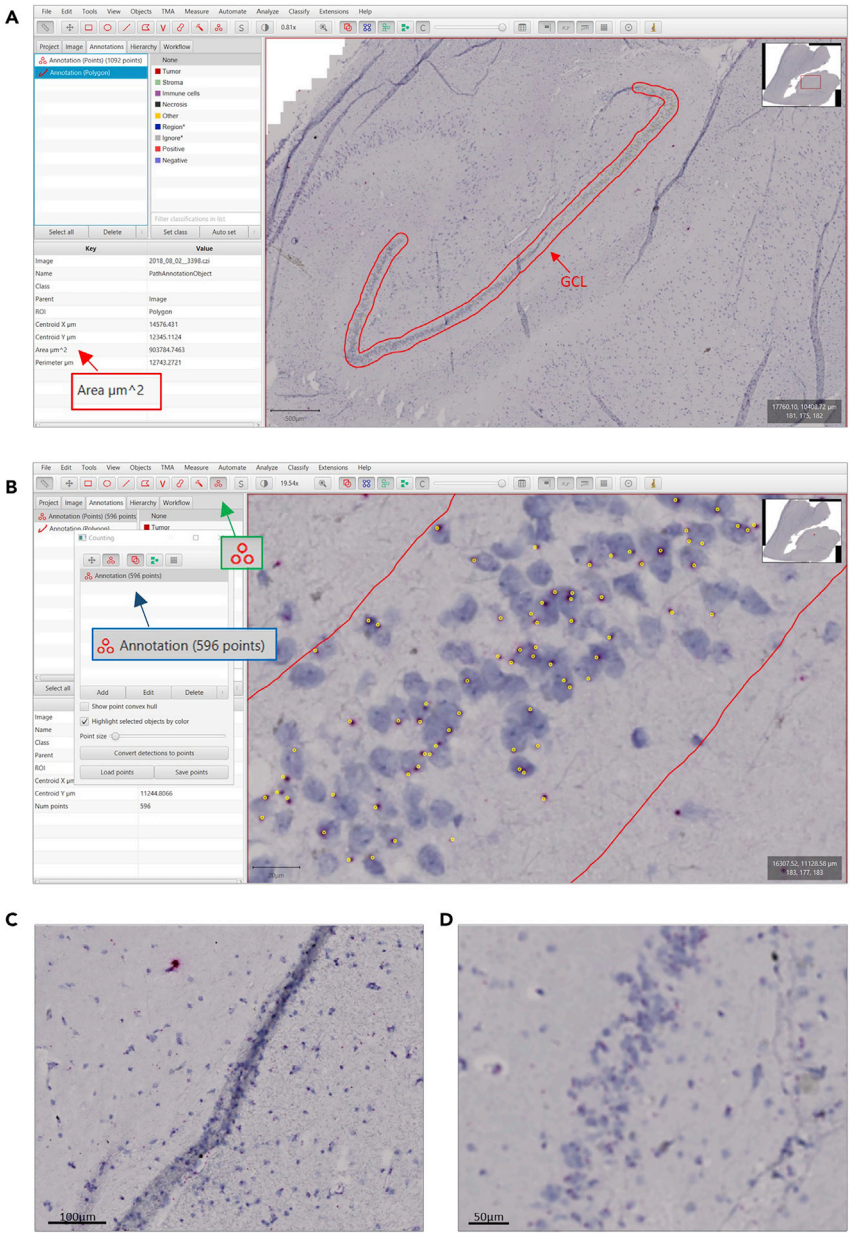


Figure 5. QuPath pipeline. Image analysis is performed using the QuPath opensource software

(A–D) First, the selection tool is used to select the ROI (A, circled in red) - in this specific case the highlighted area is the granule cell layer (GCL) of the hippocampus. The software automatically detects the size in μm^2 of the area selected (left, red arrow). When zooming in on the ROI, we used the selection tool (B, green arrow) to select the PPIB dots, that appear as a mask on top of the tissue and are automatically counted by the software (B, blue arrow). Areas that appeared out of focus (C) or folded (D) were excluded from the analysis.

Normalisation 2

Since this protocol is optimized on archived tissue, it is possible that the housekeeper count presents high variability among samples (donors), with better quality tissue having significantly more housekeeper dots (e.g., reflecting better RNA preservation state) compared to lower quality tissue. For instance, the length of the post-mortem interval (pmi) as well as the number of antemortem events (coma, respiratory illness, hospitalization, artificial ventilation, etc.) may significantly affect the pH and RNA quality of the tissue (Durrenberger et al., 2010). Moreover,

Table 2. Example of data from Hornsby et al. (2020)

	Group	Age	pmi (h)	GOI dots[a]	ROI[a] (μm^2)	GOI/μm^2	PPIB dots	ROI (μm^2)	PPIB/μm^2	Normalized value
1	CTR	93	9	745.33333	1086098.2	0.00068777	1483	504344.9	0.002940448	0.23390
2	CTR	65	12	29.333333	6088020.8	0.000004850	112	5762982.9	0.0000194344	0.24958
3	PD	76	14	346.66667	1135267.5	0.00030202	1590	989066.7	0.001607576	0.18787
4	PD	85	17	775.33333	1675322.8	0.000464159	2564	1628573.8	0.001574384	0.29482
5	PDD	82	14	304.33333	2813860.6	0.000279875	721	335988.9	0.002145904	0.13042
6	PDD	81	22	651.66667	5772784.8	0.001287096	6664	1518705.0	0.004387949	0.29333

[a]Average of 3 sections.

differences in the age and the stage of the neurodegenerative state at death, may affect the number of total cells in the tissue.

Samples with lower RNA quality exhibit a lower housekeeping signal (fewer dots) and a proportionally lower signal for the gene of interest (GOI). In order to control for these technical variations, we recommend normalizing by dividing the number of GOI dots by the PPIB dots of the same sample (donor), ultimately obtaining comparable values among the samples. Table 2 shows data extracted from our publication (Hornsby et al., 2020); samples 1 and 2 are post-mortem tissue from age-matched controls (healthy donors), samples 3 and 4 are PD donors, while 5 and 6 are PDD donors (advanced neurodegeneration and dementia).

LIMITATIONS

We applied this protocol on formalin-fixed, paraffin-embedded (FFPE) tissue, by including a xylene de-paraffination step in place of the extra-fixation step (this should not be required due to better mechanical resistance of the tissue). However, we were not able to detect any signal on FFPE hippocampal tissues from healthy or diseased subjects, despite the allegedly higher RNA quality that this type of fixation should have. Further studies should investigate whether the fixative or the xylene treatment may have damaged the RNA quality in our tissue. Indeed, formaldehyde cross-links the lysine residues, immobilising the proteins and preventing tissue decay, but also reducing reagents access to the tissue, possibly making the tissue unusable for this application.

TROUBLESHOOTING

Problem 1

Poor stain quality.

The quality and processing of the tissue may influence the outcome of the assay. When first approaching this protocol, it is recommended to optimize the key steps, according to the characteristics of the tissue used. The three main variability factors in the protocol are the target retrieval (step 9), the protease (step 14) and the AMP7 (AMP5 in the previous BaseScope™ Assay version – see Figure 3).

Potential solution

Antigen retrieval optimisation: The number of dots appearing on the tissue can be modulated by changing the timings of the pre-treatment steps, in particular the target retrieval (step 9). The positive control probe that we selected, PPIB, is a moderately expressed housekeeper (Human Protein Database, n.d.). To better understand its expression level in our tissue, we applied antigen retrieval using different timings (Figures 2D–2F). With 5 min antigen retrieval, our tissue showed very low to no-expression of the positive probe, suggesting the treatment was insufficient (Figure 2D). The second test, 15 min antigen retrieval treatment, better represented PPIB expression on our tissue (Figure 2E), compared to the levels reported in the human protein atlas (Human Protein Database, n.d.). The third option, a 30 min antigen retrieval treatment, caused the PPIB dots to form clusters that are

very difficult to count (Figure 2F). Therefore, we concluded that the 15 min antigen retrieval step was the most appropriate for our tissue, and we applied this condition to all our experiments.

Protease optimisation: The BaseScope™ Assay kit includes three proteases: III, IV and *plus*. With our brain tissue samples, we did not detect any significant differences between the three proteases (data not shown); therefore, in our experiments we only used protease IV, as it is the recommended choice for brain tissue. Protease *plus* may perform better with thicker tissue sections, while protease III is milder, therefore, it may help reduce strong tissue background. We recommend referring to the supplier's protocol for the most adequate protease to use.

AMP7 step optimisation: The incubation time of the AMP7 step can be increased to enhance the signal. However, in our samples we did not detect any significant change in signal intensity with 30- or 45-min incubations (data not shown). Optimisation of this and other time-sensitive steps, should always be performed using a negative control probe to prevent increase of non-specific signal.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the Lead Contact, Dr. Jeffrey Davies (jeff.s.davies@swansea.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All datasets generated during this study have been analysed in Hornsby et al. (2020) (https://doi.org/10.1016/j.xcrm.2020.100120).

ACKNOWLEDGMENTS

The authors specially thank former ACDbio technical support scientists Morgane Rouault and Bradley Spencer-Dene for the technical training and troubleshooting during the early stages of the optimization of this protocol. This study was funded by BRACE dementia research.

AUTHOR CONTRIBUTIONS

M.C.C. optimised this protocol during her doctoral studies, created the figures, and wrote the manuscript. O.W.H., A.H.M., and J.S.D. supervised the study. All authors discussed the results, assisted with protocol development, and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

(2020). ACDbio Analysis Guidelines. https://acdbio.com/image-analysis.

Bankhead, P., Loughrey, M.B., Fernández, J.A., Dombrowski, Y., McArt, D.G., Dunne, P.D., McQuaid, S., Gray, R.T., Murray, L.J., Coleman, H.G., and James, J.A. (2017). QuPath: opensource software for digital pathology image analysis. Sci. Rep. *7*, 16878.

Durrenberger, P.F., Fernando, S., Kashefi, S.N., Ferrer, I., Hauw, J.J., Seilhean, D., Smith, C., Walker, R., Al-Sarraj, S., Troakes, C., et al. (2010). Effects of antemortem and postmortem variables on human brain mRNA quality: a BrainNet Europe study. J. Neuropathol. Exp. Neurol. *69*, 70–81.

Hornsby, A.K.E., Buntwal, L., Carisì, M.C., Santos, V.V., Johnston, F., Roberts, L.D., Sassi, M., Mequinion, M., Stark, R., Reichenbach, A., et al. (2020). Unacylated-ghrelin impairs hippocampal neurogenesis and memory in mice and is altered in Parkinson's dementia in humans. Cell Rep. Med. *1*, 100120.

(2020). Human Protein Database. https://www.proteinatlas.org/ENSG00000166794-PPIB/tissue.

Wang, F., Flanagan, J., Su, N., Wang, L.-C., Bui, S., Nielson, A., Wu, X., Vo, H.-T., Ma, X.-J., and Luo, Y. (2012). RNAscope: a novel in situ RNA analysis platform for formalin-fixed paraffin-embedded tissues. J. Mol. Diagn. *14*, 22–29.