ALLEN INSTITUTE for BRAIN SCIENCE

Aging, Dementia and TBI Study

TECHNICAL WHITE PAPER: TISSUE PROCESSING

Overview

Inclusions of misfolded proteins build up in the brain during both normal aging and the progression of adult-onset dementias such as Alzheimer's disease. These pathologies, most notably β-amyloid, tau, and α-synuclein pathologies, have also been implicated in severe Traumatic Brain Injury (TBI)[1-6], including Chronic Traumatic Encephalopathy (CTE). The aim of the project was to allow the coordinated analysis of neuropathological markers with molecular measurements including the transcriptome and targeted proteins and nucleic acids in the same brains from a well characterized patient cohort. Some measurements, such as immunohistochemical analysis of neuropathological markers, were performed on fixed tissue specimens, whereas others, including transcriptomics and *in situ* hybridization, required the use of fresh frozen unfixed tissue.

To accurately assess the potential effects of mild-moderate TBI in our aged cohort, fixed tissue was stained by immunohistochemistry (IHC) and histochemistry to assess β-amyloid, tau, and α-synuclein deposition in plaques, tangles, and Lewy bodies, respectively using Formalin-Fixed Paraffin-Embedded (FFPE) preparations standard in the field. To more accurately correlate local pathology and transcriptome variation as they relate to aging, dementia and mild to moderate TBI, it was important to measure pathology and RNA expression in the same tissue blocks. Fresh frozen tissue blocks were therefore processed both for histological assessment (to allow for a comparison to the neuropathology information obtained from the fixed tissue) and RNA sequencing. In addition, this fresh frozen tissue was used for *in situ* hybridization (ISH) for canonical marker genes for major cell classes to gain insight into the cellular makeup of the tissue. For IHC of FFPE tissues, blocks were taken from parietal cortex and temporal cortex from the same side of the brain that was sampled during the rapid autopsy for frozen tissues. FFPE hippocampus for IHC was taken from the opposite side as the entire hippocampus hemisphere was used for frozen tissue processing (see accompanying Tissue Collection Technical White Paper in the Documentation tab).

FIXED TISSUE PROCESSING

Tissue Sectioning

FFPE sections were cut at 5 µm thickness with Sakura Accu-edge low profile microtome blades on Leica RM2245 microtomes (see **Figure 1** for sectioning schematic). Sections were floated on a warm water bath to help remove wrinkles, and then picked up on glass microscope slides (Leica Apex Superior Adhesive slides 1" x 3" x .04"), blotted, and baked for two hours at 65°C in preparation for staining.

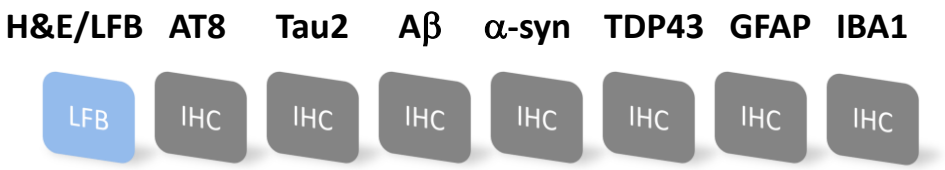

Figure 1. Sectioning schematic for formalin-fixed paraffin-embedded tissue blocks.
Five-micron thick sections were obtained from each formalin-fixed paraffin-embedded tissue block and designated for staining. Sections were collected for histological staining (hematoxylin and eosin-Luxol Fast Blue, H&E/LFB) and immunohistochemistry (IHC) (see **Table 1** for antibody details). IHC stains include phospho-tau (immature and mature) (AT8), phospho-tau (mature) (Tau2), beta-amyloid (Aβ), α-synuclein (α-syn), phospho-TDP43 (TAR DNA binding protein) (TDP43), glial fibrillary acidic protein (GFAP), and ionized calcium binding adaptor molecule 1 (IBA1), which is also known as allograft inflammatory factor 1 (AIF-1).

Tissue Processing

Immunohistochemistry-Formalin Fixed
For the detection of antigens through the use of monoclonal and/or polyclonal antibodies on FFPE tissue sections (see **Table 1**), an automated procedure was used based around the Bond Polymer Refine Detection system (Leica Biosystems). This novel controlled polymerization technology was used to prepare polymeric horseradish peroxidase (HRP)-linker antibody conjugates. This detection system avoids the use of streptavidin and biotin, and therefore eliminates non-specific staining as a result of endogenous biotin.

The Bond Polymer Refine Detection (Leica Biosystems) works as follows:

- The specimen is incubated with hydrogen peroxide to quench endogenous peroxidase activity;
- A user-supplied specific primary antibody is applied;
- Post primary IgG linker reagent localizes mouse antibodies;
- Poly-HRP IgG reagent localizes rabbit antibodies;
- The substrate chromogen, 3, 3'-diaminobenzidine tetrahydrochloride (DAB), visualizes the complex via a brown precipitate;
- Hematoxylin (blue) counterstaining allows for visualization of cell nuclei.

This automated procedure also includes dewaxing, epitope retrieval, and washes. Once complete, slides were removed, dehydrated through sequential washes through increasing ethanol concentrations and finally in xylenes prior to coverslipping with Micromount (Leica Biosystems).

Tissue sections (fixed in 10% neutral buffered formalin) were cut at 5 µm, mounted on positively charged 1” x 3” slides, and baked for 2 hours at 65ºC. One positive control section (severe Alzheimer's disease brain) was included for each antibody run.

The following reagents were utilized:

- Bond™ Dewax Solution: deparaffinization solution
- Peroxide block: 3-4% hydrogen peroxide
- Post primary: rabbit anti-mouse IgG (<10µg/mL) in 10% (v/v) animal serum in Tris-buffered saline/0.09% ProClin™ 950
- Polymer: anti-rabbit poly-HRP-IgG (<25µg/mL) containing 10% (v/v) animal serum in Tris-buffered saline/0.09% ProClin™ 950
- DAB Part 1: 66mM 3,3'-diaminobenzidine tetrahydrochloride, in a stabilizer solution
- DAB Part B: ≤0.1% (v/v) hydrogen peroxide in a stabilizer solution
- Hematoxylin: 0.1% Hematoxylin
- Epitope Retrieval Solution 1 (ER1): citrate based buffer and surfactant (pH 5.9-6.1)
- Epitope Retrieval Solution 2 (ER2): EDTA (ethylenediaminetetraacetic acid) based buffer and surfactant (pH 8.9-9.1)
- Enzyme Pretreatment: Dilute to three different strengths as either:
    - Enzyme 1 (ENZ1): 1 drop enzyme concentrate, 7 mL of enzyme diluent

  - Enzyme 2 (ENZ2): 2 drops enzyme concentrate, 7mL of enzyme diluent
- Enzyme Concentrate: proteolytic enzyme and stabilizer
- Enzyme Diluent: Tris-buffered saline, surfactant, and 0.35% ProClin™ 950
- Antibody Diluent: Tris-buffered saline, surfactant, protein stabilizer and 0.35 % ProClin™ 950

Table 1. Antibodies and pretreatments utilized for IHC on fixed tissue.

Primary Antibody	Clone	Mono or Poly	Pretreatment**	Antibody Dilution 1:X	Raised In	Manufacturer	Control Tissue
Phospho-PHF-tau*		Mono	ER2 (30 min)	1,000	Mouse	Pierce	Hippocampus/AD
TAU 2	tau 2	Mono	ER2 (10 min)	5,000	Mouse	Sigma-Aldrich	Hippocampus or cortex/AD
A Beta (β-amyloid)	6E10	Mono	ER2 (20 min)	2,000	Mouse	Covance	Cortex/AD
α-synuclein	LB509	Mono	ENZ1 (10 min)	500	Mouse	Invitrogen	Amygdala or cortex/LBD
TDP-43 Ser409/Ser410	1D3***	Mono	ER2 (20 min)	250	Rat	Millipore	Hippocampus/HS
GFAP		Poly	ER1 (20 min)	10,000	Rabbit	Dako	Cortex/DZ
IBA1		Poly	ER2 (20 min)	1,700	Rabbit	Wako	Cortex/DZ

Abbreviations: AD, Alzheimer's disease; DZ, any neurodegenerative disease; HS, Hippocampal sclerosis; LBD, Lewy body dementia; PHF, paired helical filament.
*The full name of this antibody is Phospho-PHF-tau pSer202+Thr205 (AT8).
**Pretreatment details are described in the above text.
***This antibody is against phospho TDP-43 Ser409/Ser410.

H&E and LFB Histology

Paraffin sections were cut at 5 µm and deparaffinized in xylene, 100% and 95% ethanol. Slides were then placed in Luxol® Fast Blue (LFB) staining solution (0.1% LFB in 95% alcohol, 0.5% of 10% acetic acid), covered with aluminum foil, and incubated overnight at 65ºC. After cooling to room temperature, slides were transferred to 95% ethanol to clear excess LFB and then rinsed with deionized H_20. Next, slides were dipped in lithium carbonate solution (0.1% lithium carbonate in deionized H_20) 10 times. Then, slides were dipped in 70% alcohol 10 times for differentiation, followed by rinsing in deionized H_20. Lithium carbonate solution incubation through rinsing in deionized H_20 was repeated until the background was clear (gray and white matter could be distinguished). After staining was determined to be sufficient, slides were rinsed in deionized H_20 for 2 min, then stained in Mayer's Hematoxylin (BBC, #3580) for 2 min, and then rinsed in deionized H_20 for 2 min. Next, the tissue was dipped in lithium carbonate solution (0.25%) 20 times for bluing, rinsed in deionized H_20 for 2 min, then stained in eosin for 2 min (working eosin stain consisted of 50 ml of 1% alcohol eosin, 5 ml of 1% phloxine B, 390 ml of 95% ethanol, and 2 ml glacial acetic acid), and rinsed in deionized H_20 for 2 min. Dehydration consisted of 95% ethanol, followed by 100% ethanol, and clearing with xylene. The slides were mounted with Micromount (Leica Biosystems). In the majority of cases, the first complete section per fixed tissue block was stained for H&E and LFB histology.

FRESH FROZEN TISSUE PROCESSING

Sectioning

Fresh frozen tissue samples from temporal cortex, parietal cortex, and hippocampus were sectioned in Leica CM3050 S cryostats (object temperature, -10°C; chamber temperature, -15°C) at 25 µm thickness in the coronal plane from anterior to posterior. The first two sections were used for RNA quality assessment of the tissue block. RNA quality assessment was performed using Ambion's MELT Total Nucleic Acid Isolation System (AM1983) according to the manufacturer's instructions. Each 25 µm section for RNA assessment was collected into 100µl of MELT Buffer/Cocktail mix, immediately vortexed to homogenize the tissue, then frozen at -80°C until RNA isolation was performed. Isolated RNA was normalized to 5ng/µl and 1µl was run on an RNA Pico chip using the Agilent Bioanalyzer 2100, generating an RNA Integrity Number (RIN). After the two sections for RNA assessment were collected, tissue was then collected onto slides. One section was placed on each positively

charged Superfrost Plus™ 1" x 3" microscope slide (Erie Scientific Co.), pre-printed with a unique identifying barcode for tracking (see **Figure 2** for sectioning schematic). Specimen numbers were also printed onto the slides for tracking purposes. Samples too large to fit on slides were trimmed prior to sectioning.

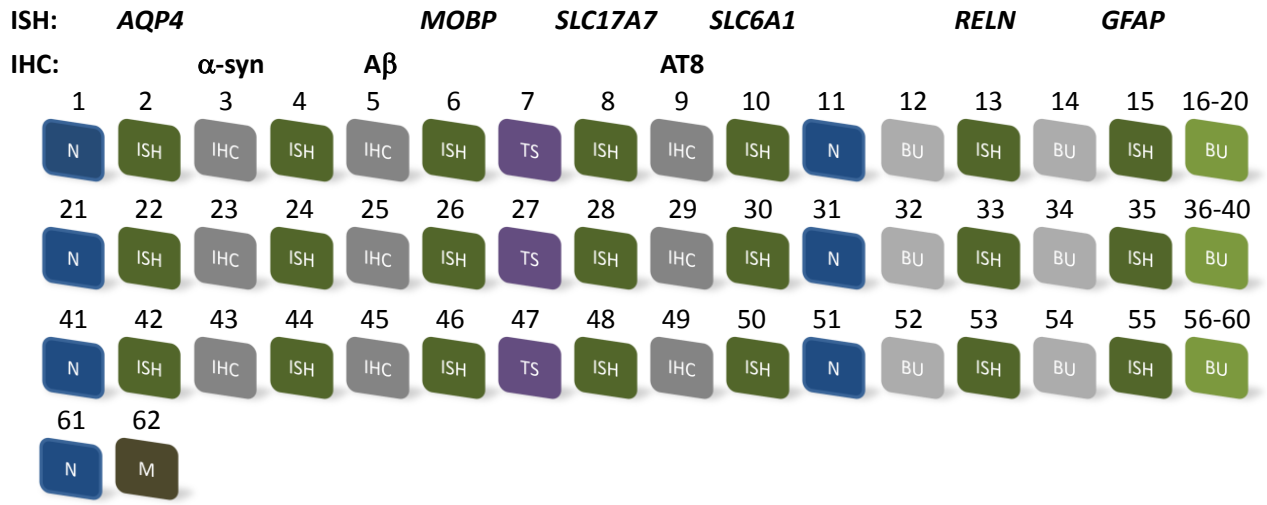

Figure 2. Sectioning schematic for fresh frozen tissue blocks.
Twenty-five-micron thick sections were obtained from each fresh frozen tissue block and designated for histological staining based on this sectioning scheme (serial section number indicated by 1, 2, 3, etc.). Sections were collected for histological staining for Nissl (N, blue), *in situ* hybridization (ISH, green), immunohistochemistry (IHC, gray), and Thioflavin-S (TS, purple). The specific gene ISH riboprobes (see **Table 3** below) and IHC stains (α-syn, α-synuclein; Aβ, β-amyloid; AT8, phosphorylated tau) are listed above each series of slides subjected to that individual stain. Back-up (BU) or additional slides were also collected for IHC and ISH (light gray and light green, respectively). The final sample collected from each tissue block was designated for macrodissection and RNA isolation for RNA sequencing (M, brown). For some tissue blocks, fewer sections were collected and the number of sections per stain was reduced.

Following sectioning, slides designated for:

- Nissl staining were stored at 37°C for 1-7 days and then processed. Up to 7 slides per tissue block were Nissl-stained.
- Thioflavin-S staining were allowed to air dry, fixed for 20 minutes in 4% neutral buffered paraformaldehyde (PFA), rinsed in 1x PBS, then dehydrated using a graded series of 50%, 70%, 95%, and 100% ethanol. Slides were stored at room temperature in Parafilm®-sealed boxes. Up to 3 slides per tissue block were subjected to Thioflavin-S staining.
- Immunohistochemistry (IHC) were allowed to air dry for 30-60 minutes before being boxed, vacuum sealed and stored at -80°C. Up to 15 slides per tissue block were designated for IHC, with up to 3 slides per IHC stain.

Following sectioning, slides designated for *in situ* hybridization (ISH) were allowed to air dry and tissue was fixed, acetylated, and dehydrated according to standard protocols as described[7]. Briefly, tissue was fixed for 20 minutes in 4% neutral buffered paraformaldehyde (PFA) and rinsed in 1x PBS (phosphate buffer saline), acetylated for 10 minutes in 0.1M triethanolamine with 0.25% acetic anhydride, and subsequently dehydrated using a graded series of 50%, 70%, 95% and 100% ethanol. Slides that passed section quality checks were stored at -80°C in Parafilm®-sealed slide boxes until use. Up to 36 slides per tissue block were designated for ISH, with 3 slides allocated for each riboprobe.

After collecting the above tissue sections, macrodissection of regions of interest was performed to isolate samples for RNA sequencing from the remaining tissue (see Quantitative Data Generation white paper, from the Documentation tab).

Histological Staining

Nissl Staining
After brain tissue was sectioned, slides for Nissl staining were stored at 37°C for 1–7 days and were removed 5–15 minutes prior to staining. Sections were defatted with the xylene substitute Formula 83 (CBG Biotech), and hydrated through a graded series containing 100%, 95%, 70%, and 50% ethanol. After incubation in water, the sections were stained in 0.213% thionin, rinsed in water and dehydrated in a graded series containing 50%, 70%, 95%, and 100% ethanol. Finally, the slides were incubated in Formula 83, and coverslipped with the mounting agent DPX (VWR #100503-834). After drying, the slides were analyzed microscopically to ensure staining quality. Slides that passed quality control (QC) were stored at room temperature in slide boxes before being cleaned in preparation for digital imaging.

Thioflavin-S
Thioflavin-S staining protocols were based on previously described methods[8]. Slides were fixed and dehydrated then stained for 5 minutes with a mixture of Thioflavin-S (Sigma; 0.125 mg/mL) and Sytox Orange (Invitrogen; 0.1 µl/10mL) in 50% ethanol. Slides were rinsed in fresh 50% ethanol and water, coverslipped using Fluoromount-G. Slides were subjected to quality control assessment and stored at room temperature prior to fluorescent imaging to visualize stained plaques (Thioflavin-S) and cell nuclei and neurites (Sytox Orange).

Immunohistochemistry

Immunohistochemical staining was used to visualize amyloid, tau, and alpha-synuclein pathology within the tissue. Fresh frozen tissue sections mounted on slides were taken out of storage at -80°C, equilibrated to room temperature, and fixed with 100% acetone chilled to approximately -20°C. Sections were then rehydrated in 1X PBS, pH 7.4 (1:10 dilution of 10X PBS, Ambion) and incubated in 10mM sodium citrate for 10 min at 98°C. Slides were allowed to cool and were then washed in PBS–Tween 20 (0.05%) before starting the staining protocol. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in PBS and slides were washed in PBS–Tween 20 before incubation in blocking solution containing 4% horse serum (Vector Laboratories) and 0.3% Triton-X in PBS. Sections were then incubated with primary antibodies (see **Table 2** for antibody information) diluted in blocking solution overnight. The following day, sections were rinsed in PBS–Tween 20 before incubation in biotinylated anti-mouse secondary antibody (1:500, Vector Laboratories, #BA-2000) prepared in blocking solution. Sections were rinsed in PBS-Tween, incubated for 30 min in ABC (Vectastain, Vector Laboratories), and the reaction product visualized with 0.05% DAB (Sigma-Aldrich), containing 0.01% hydrogen peroxide. Sections were washed a final time in PBS, dehydrated through graded alcohols, cleared with Formula 83, and coverslipped with DPX. Slides were subjected to quality control assessment and stored at room temperature prior to digital imaging.

Table 2. Antibody information for IHC on fresh frozen tissue.

	Beta-amyloid	Phosphorylated tau	Alpha-synuclein
Antibody	Ab6e10	AT8	LB509
Source	Beta Amyloid-Mouse Biolegend Cat #SIG-39320	Tau AT8-Mouse Pierce Cat #MN1020	Alpha Synuclein-Mouse Life Tech Cat #18-0215
Pre-treatment	Acetone Fix, Sodium Citrate	Acetone Fix, Sodium Citrate	Acetone Fix, Sodium Citrate
Primary antibody	1:2000	1:2000	1:2000
Secondary antibody	1:500	1:500	1:500
ABC	45µl/10mL	45µl/10mL	45µl/10mL
DAB	1 min	1.5 min	7 min
Post-staining	Dehydrate, DPX	Dehydrate, DPX	Dehydrate, DPX

Colorimetric *In Situ* Hybridization

It is widely known that Alzheimer's diseases and other dementias are associated with alterations in cell type-specific function, including gliosis and neuronal dysfunction and cell death. To examine the cellular makeup of tissues used for neuropathological and transcriptomic analysis, a colorimetric, digoxigenin-based method for labeling target mRNA was used to detect expression for canonical marker genes for astrocytes (*AQP4*, *GFAP*), oligodendrocytes (*MOBP*), and neuronal subtypes (**Table 3**). Specifically, the vesicular glutamate transporter gene *SLC17A7* was used to label all excitatory neurons, the GABA transporter *SLC6A1* to label all inhibitory interneurons, and Reelin (*RELN*) to label selective inhibitory neuronal populations. Three slides per tissue block were hybridized to each ISH riboprobe.

Table 3. Genes selected for ISH.

Gene Symbol	Gene Name	Entrez Gene ID	Target Factors
AQP4	aquaporin 4	361	All astrocytes
GFAP	glial fibrillary acidic protein	2670	Activated astrocytes
MOBP	myelin-associated oligodendrocyte basic protein	4336	Mature oligodendrocytes *Age*
RELN	reelin	5649	Interneuron subtype *pTau load*
SLC6A1	solute carrier family 6 (neurotransmitter transporter), member 1	6529	Inhibitory cells (GABA transporter) *pTau load*
SLC17A7	solute carrier family 17 (vesicular glutamate transporter), member 7	57030	Excitatory cells (VGLUT1) *Ab_{42}, pTau load, Dementia status*

Probe Design and Synthesis
For labeling target mRNA in tissue sections using ISH, digoxigenin-labeled riboprobes were designed and synthesized according to specific criteria. Briefly, using sequences obtained from RefSeq and a semi-automated process based on Primer3 software (Rozen and Skaletsky, 2000), probes were designed to be between 400-1000 bases in length (optimally > 600 bases) and to contain no more than 200 bp with > 90% homology to non-target transcripts. In addition, to allow comparability of mouse and human gene expression datasets, when possible human probes were designed to have > 50% overlap with the existing Allen Mouse Brain Atlas probe when the mouse and human genes were orthologous. Riboprobes were synthesized using standard *in vitro* transcription (IVT) reactions based on PCR templates prepared from human cDNA clones (NIH Mammalian Gene Collection, Open Biosystems, Huntsville, AL) or pooled cDNA synthesized from human brain total RNA. cDNA was prepared from human brain RNA from prefrontal, temporal, parietal, occipital, and frontal cortical areas as well as medulla and cerebellum (Ambion, Austin, TX) using Superscript III RTS First- Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), then pooled in equal amounts to provide templates for PCR.

PCR primers were obtained from Integrated DNA Technologies (Coralville, IA) at a final concentration of 10 μM, and designed with GC content between 42%–62% and an optimal size of 22nt with lower and upper limits of 18nt and 26nt, respectively. For cDNA clones, the clone sequence was compared with RefSeq sequences, and consensus sequences with >98% homology across 80% of the total length were used to develop probes. When a clone was used as a template, a single PCR was used requiring only a forward and reverse primer with an additional SP6 RNA polymerase binding sequence (GCGATTTAGGTGACACTATAG). When using brain cDNA as a template, probes were generated against sequences within a region 3,000 bp from the 3' end using 3 primers: forward, reverse, and a nested reverse primer containing the SP6 RNA polymerase binding sequence. cDNA primers underwent a BLAST analysis to verify amplification of only target sequence. All cDNA reactions were run on the Bioanalyzer for quality control.

Standard conditions for PCR and IVT reactions were as described[7]. IVT reactions were diluted to working stocks of 30 ng/µl with THE (0.1mM Sodium Citrate pH 6.4, Ambion). Aliquots were stored in one- or two-use volumes to minimize freeze/thaw cycles. IVT dilutions were stored at -80°C. For hybridization, the probe was diluted 1:100 (to 300ng/ml) into *in situ* hybridization buffer (Ambion) in 96-well ISH Probe Plates (GFAP probes were diluted 1:200 (to 150 ng/ml)). Each well provides probe for one ISH slide. Probe plates were stored at -20°C until used in an ISH run.

All PCR and IVT products were run on the Bioanalyzer for size and morphology quality control. Specifically, PCR products that were not of the correct size (+/- 100bp) or that showed multiple products were not used to generate riboprobes. IVT products that were shorter than their predicted size were not used. It is common to see IVT products that run slightly larger than their predicted molecular weight, or as multiple peaks, due to secondary structure of the RNA. IVT products with multiple bands were not used for ISH unless the additional bands were determined to result from secondary structure.

High-Throughput In Situ Hybridization
ISH processes were performed on robotic platforms that allowed processing of 192 1" x 3" slides at a time. Detailed descriptions of the high-throughput platform, protocols, and reagent preparation are as described[7]. In general, slides containing tissue sections were placed in flow-through chambers on temperature-controlled racks on computer-controlled Tecan EVO liquid handling platforms for addition of solutions. The first steps blocked endogenous peroxidase activity and permeabilized the tissue (0.0175U/ml Proteinase K), followed by subsequent hybridization of digoxigenin-labeled probes to target mRNA. After a series of washes to eliminate excess probe, the remaining bound probe was subjected to a series of enzymatic reaction steps to detect and amplify digoxigenin signal. First, a horseradish peroxidase (HRP)-conjugated anti-digoxigenin antibody (0.10 U/ml) was added, followed by biotin-coupled tyramide (TSA-Plus™, Perkin Elmer, Waltham, MA) that is converted by HRP to an intermediate that binds to cell-associated proteins at or near the HRP-linked probe. TSA-Plus (1.5 µl/slide) was used for the GFAP probe, while the other five probes received 2.0 µl/slide of TSA-Plus. Neutravidin conjugated with alkaline phosphatase (AP) was then bound to biotin and BCIP/NBT was added. A blue/purple particulate precipitate forms as a result of the enzymatic cleavage of BCIP by AP and subsequent indole reaction with NBT. Finally, the colorimetric reaction was stopped by washing with EDTA (ethylenediaminetetraacetic acid) and fixed with 4% PFA. This entire process occurred over the course of approximately 23.5 hours on the Tecan automated platform.

To reduce background signal, an acid alcohol wash step was performed after completion of the hybridization process. Slides were rinsed 4 times (1 min each) in acid alcohol (70%, adjusted to pH = 2.1 with 12N HCl) and rinsed 4 times in Milli-Q® water (1 min each). Acid alcohol and water solutions were refreshed every fourth rack to ensure that all slides were rinsed in clean solution.

Each ISH run included several controls. Two positive controls in mouse tissue (*Drd1a* and *Calb1*) were used to provide verification of a successful ISH run, and a negative control (no probe) in mouse tissue was included as an indication of background for each ISH run. Human *GAP43* and *CALB1* were included in all experiments to gauge signal from a probe with typically high and moderate expression, respectively. Both probes were run on banked tissue from the human visual cortex.

Image Acquisition
Colorimetric ISH and other histologically stained whole slides were scanned using the Leica ScanScope® automated slide scanner (Leica Biosystems). The line scan camera continually adjusts for focus based on a variable number of focus points set by the user for imaging large tissue sections that tend to have variation in focal plane. Images were acquired with at 10x magnification (objective lens 20x/0.75 NA Plan Apo) resulting in a pixel resolution of approximately 1.0 um/pixel in SVS file format using ScanScope Console (version 101.0.0.18) and controller (version 101.0.4.446).

Thioflavin-S stained slides used to identify amyloid plaques were scanned on a semi-automated, high-throughput fluorescence scanning system VS110 or VS120 (Olympus) using a 10x objective (N/A 0.4) and a UV/DAPI Longpass filter (Chroma). Resulting images have a pixel resolution of approximately 0.64 µm/pixel.

During review of all images, the automated bounding box overlay was manually adjusted and placed over the corresponding tissue section. Images were inspected and re-scanned if artifacts such as out of focus and image tile stich misalignments compromised the quality of the images. Images were failed if data analysis was compromised by artifacts (*e.g.* mechanical damage, mounting medium bubbles, hybridization bubbles, and NBT/BCIP precipitated aggregates) associated with the corresponding tissue section.

Image Data Processing Pipeline

Once images were acquired, an Informatics Data Pipeline (IDP) managed image preprocessing, image QC, IHC expression detection and measurement, Nissl processing, annotation QC and public display of information via the Web application. The IDP has been described in detail previously[9], with some modifications and additions for processing images for this project.

Nissl, H&E-LFB, ISH and IHC slides were scanned at 10x full resolution using a Leica ScanScope scanner, while Thioflavin-S slides were scanned at 10x full resolution using an Olympus VS110 scanner (as described above). The images were first converted to the JPEG 2000 format and then oriented for consistency across slides. Except for the Thioflavin-S slides, all images were also white balanced to achieve consistent white background intensities.

For the ISH slides, masks highlighting areas with enriched gene expression were generated using adaptive detection/segmentation image processing algorithms. Similar techniques were used with IHC slides to obtain masks highlighting areas with enriched immunoreactivity. Due to the presence of blue in the signal background, the mask calculation for FFPE IHC slides was preceded by a spectral filtering step.

Finally, all images were converted to the AFF format for Web display.

QUALITY CONTROL AND PUBLIC DISPLAY

After image acquisition, and during the course of data processing, two additional quality control steps occurred. Once image preprocessing was complete, image quality control ensured focus criteria were met and provided an initial indication of the presence of signal. If focus criteria were not met, the images were failed and the appropriate slides were rescanned. If focus criteria were met, the images were passed and proceeded through the IDP for further processing.

A second phase of quality control comprised verification of anatomic region and verification that gene expression data were not obscured or adversely affected by technical or tissue artifacts. Images that met anatomic and expression detection criteria were passed for public release. For each set of gene images available in the online viewer, the nearest set of Nissl-stained sections (and other histological data) can be accessed and viewed.

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