ALLEN INSTITUTE for BRAIN SCIENCE
Fueling Discovery

ALLEN **Mouse Brain Atlas**

TECHNICAL WHITE PAPER: *IN SITU* HYBRIDIZATION DATA PRODUCTION

OVERVIEW

The processes and protocols used by the Allen Mouse Brain Atlas (the Atlas) were designed based upon the methods developed by Dr. Gregor Eichele's laboratory at the Max Planck Institute and Baylor College of Medicine. A state-of-the-art facility was constructed for performing *in situ* hybridization (ISH) in a highly consistent, automated, industrialized fashion. The data production laboratory was designed with specifications to allow full-capacity production of approximately 1,000 slides, accommodating 4,000 mouse brain sections, daily. The facility has strict environmental controls on air humidity and temperature as well as an RNAse-free water system capable of delivering the 300 liters of water necessary to run at least five robotic *in situ* hybridization systems daily.

The Allen Institute for Brain Science has developed a Laboratory Information Management System (LIMS) to organize and track the steps involved in creating quality ISH data. Bar codes are used to track reagents and samples and an automated system is used for work planning and recording of quality control parameters. The output quality of the Atlas platform is maintained by established metrics for success/failure at each step in the process. All processes associated with data production, including solution preparation, probe preparation, ISH, equipment maintenance, animal care and other laboratory maintenance functions are governed by Standard Operating Procedures (SOPs). These SOPs are revision-controlled and changes to these procedures are reviewed and validated prior to implementation.

The lab operates in a mode of continuous process and automation improvement. The standards for data quality continue to be evaluated and elevated. To that end, certain processes have been modified during the evolution of the platform, to take advantage of technological advances and refine protocols based upon on our own experience and other published work.

The Atlas production processes are summarized in the flow chart below. The remainder of this document discusses these steps in detail. The full protocol for the *in situ* hybridization process can be found in Appendices 1 and 2.

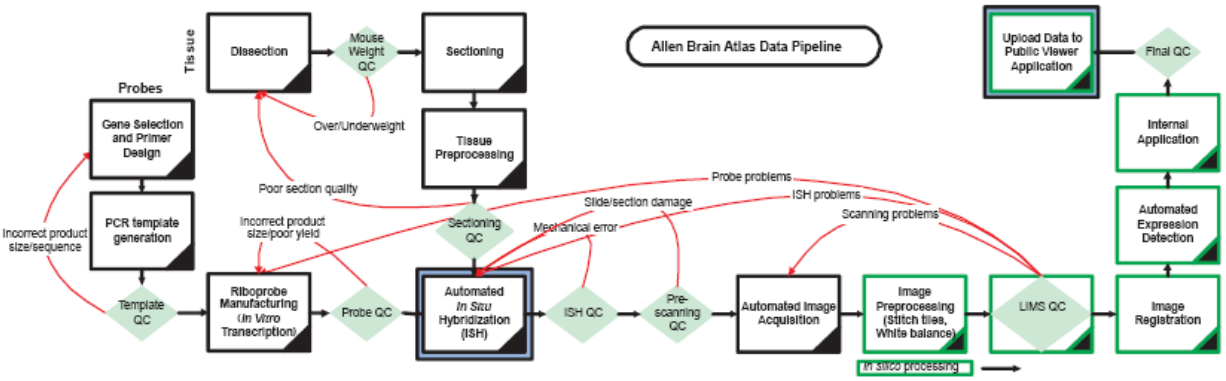

Figure 1. Production process.

GENE SELECTION AND RIBOPROBE SYNTHESIS.

The Atlas contains ISH data for approximately 20,000 distinct mouse genes. The workflow and methods used for generating riboprobes are as follows:

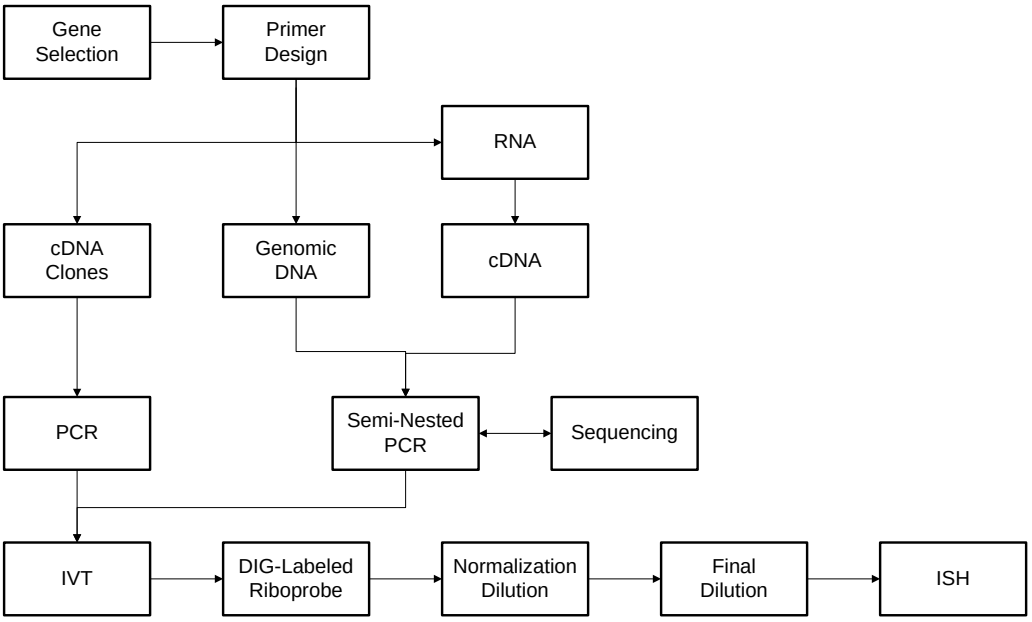

Figure 2. Probe production workflow.

Abbreviations: PCR, polymerase chain reaction; IVT, in vitro transcription; DIG, digoxigenin; RNA, ribonucleic acid; DNA, deoxyribonucleic acid. cDNA, complementary DNA.

Gene selection
The initial approach for gene selection was to target blanket coverage of unique entries in the RefSeq database. This collection was later enlarged to include sequences from TIGR and Celera databases, as well as the Riken FANTOM3 clone collection.

Probe design
A semi-automated process was used for probe design. Sequences were obtained from multiple sources including RefSeq, MGC, Celera, TIGR, FANTOM3/Riken, and UniGene. One of three sources of DNA were used as templates for PCR: cDNA clones (MGC or Riken), pooled cDNA from mouse brain, or genomic tail DNA.

cDNA clones
Clones were used as direct templates for PCR. Clones were stored as glycerol stock in 384-well and 96-well plates at -80˚C. Approximately 9,000 clones from MGC (Mammalian Gene Collection, NIH) and 2500 clones from FANTOM3 (Riken) have been used to date. When cDNA clones are available the clone sequence is compared with RefSeq sequences. Only clones with consensus sequences with >98% homology to RefSeq transcripts were used for probe designs. 80% of the total length are used to develop probes.

cDNA templates
When clones are unavailable for a given gene, pooled cDNA reactions made from mouse brain total RNA were used as a template source. Probes were generated against sequences within a region 3000 bp from the 3' end of cDNA. Approximately 9000 probes have been generated using cDNA as a PCR template.

Mouse brain cDNA preparation
Total RNA was isolated from homogenized wild type C57BL/6J mouse brains using the ToTALLY RNA kit (Ambion) per the manufacturer's protocol. Total RNA was visualized on a Bioanalyzer and quantified by A260 readings using a SpectraMaxM2 plate reader (100 µl at a 1:50 dilution). Typical yield was 120 µg total RNA from one brain. The Superscript III RTS FirstStrand cDNA Synthesis Kit (Invitrogen) was used for cDNA reactions. Reactions were performed in a 96-well plate per manufacturer's directions, using 5 µg of Anchored oligodT25. Each brain supplied enough RNA for 2x20 µl reactions. cDNA reactions were pooled from each brain (480 µl), sufficient to supply template material for 4x96 PCR reactions. Twelve samples from each 96 well cDNA reaction plate were run on the Bioanalyzer for quality control.

Genomic DNA
Genomic DNA was isolated from mouse tail snips using either DNAeasy Tissue Kit (Qiagen) or Xtractor DNA kit (Qiagen). Tail clips from two C57BL/6J mice (0.6 cm each) were combined for each DNA isolation reaction. DNA was run on a gel to confirm that only high molecular weight DNA was present (nothing visible below 500 bp). When using genomic DNA as a template, probes were designed within single exons with a minimal length of 400 bp. Approximately 1000 probes were generated using genomic DNA as a PCR template.

Primer design
All gene sequences were analyzed by BLAST against the entire collection of transcript sequences described above. Regions of homology greater than 80% (formerly 70%) for regions over 200 bp (formerly 100 bp) were identified and excluded for probe design. However, for a subset of genes in families with high homology, these standards were necessarily relaxed to >90% for regions >120 bp. Within the remaining sequence, primers were designed using Primer3 software (MIT). When a cDNA clone was used as a template, only a single PCR reaction was necessary, and therefore only a forward and reverse primer were used. When mouse brain cDNA or genomic DNA were used as PCR templates, a nested approach was used for the generation of probes. In this case, three primers were generated: a forward, a reverse, and a nested primer. The transcript is initially amplified by PCR using the forward and reverse primers, and the product is then used as a template for a second round of PCR using the same forward primer and the nested primer.

Gene-specific forward and reverse primers were designed according to the following protocol. Stricter criteria for probe design have been implemented over time, and are noted where appropriate.

1. BLAST analysis was performed to find regions of low homology between the transcript sequence and other genes/gene family members.
2. Repetitive and/or homologous sequences were masked out to avoid cross-hybridization to other genes (described below).
3. Primer3 software was used for primer design with specific criteria:
   a. The optimal primer size was 22 nt, with a minimum length of 20 and maximum length of 24.
   b. The GC content was between 42-52%.
   c. The product size was between 400-1200 nt (formerly 300-1200 nt) with an optimal size of at least 600 nt. The current standard for probe design sets a minimum of 400 nt for the probe.
   d. Probe location within gene:
      i. Clone templates were used when available (no bias of location of probe sequence within clone sequence).
      ii. For cDNA templates, the probe was designed within 3000 nt of the polyA tail.
      iii. For Genomic templates, the probe was designed within a single exon.
   e. The top primer pair meeting these criteria is chosen.

4. For cDNA and genomic DNA templates (but not clone templates), a nested reverse primer was also designed to ensure specificity of the amplified probe.
5. To the reverse (or nested) antisense primer, an SP6 RNA polymerase binding sequence (GCGATTTAGGTGACACTATAG) was added.
6. Primers were ordered from IDT in the 96-well format and delivered at 10 µM final concentration.

PCR

Standard PCR reactions were performed using Qiagen Taq Polymerase. All reactions were run in 96-well format for 35 cycles, 50 µl total volume with final concentrations of 1.5 mM MgCl2 (1x Taq buffer), 0.5 µM oligonucleotide primers (IDT), 200 µM dNTPs (Roche), and 1.25 U Taq Polymerase. Clone glycerol stock, cDNA pool, or genomic DNA was used as template material (1.0 µ). A second round of PCR using the nested reverse primer was performed for cDNA and genomic DNA templates. PCR reactions were purified using the Montage 96 filter plate (Millipore)per the manufacturer's protocol, and eluted with 50 µl of 10 mM Tris pH8.0 following a 30 min room temperature incubation. PCR reactions were quantified by A260 readings using a SpectraMaxM2 plate reader (100 µl at 1:25 dilution). Each PCR reaction was run on Bioanalyzer 2100 (Agilent) (at 1:2 dilution) for product size confirmation and quantification. PCR reactions were stored at -20˚C.

Sequencing

A large subset of the PCR products generated from cDNA and genomic DNA templates were sequenced from both ends, using the forward primer and Sp6. This sequencing step was used to confirm that the probe design process was generating the expected probe sequence. Sequencing was done on MegaBACE and ABI3700 capillary instruments by htSEQ (www.htseq.org, previously Rexagen).

In vitro transcription

Standard in vitro transcription (IVT) reactions were performed using the 10x DIG RNA Labeling Mix (Roche). All reactions were done in 96-well format for 2 hours at 37˚C, 30 µl total volume with final concentrations of 1x DIG labeling mix and 1x Transcription Buffer (NEB), containing 60U Protector RNase Inhibitor (Roche) and 60U Sp6 RNA Polymerase (NEB). Purified PCR product (12 µl, approximately 600-1200 ng) was used as template material. IVT reactions were purified using the Montage 96 filter plate (Millipore) per the manufacturer's directions, and eluted with 90 µl of THE (0.1 mM Sodium Citrate pH 6.4, Ambion) following 30 minute room temperature incubation. IVT reactions were quantified using the RiboGreen HIGH assay (molecular Probes) and the SpectraMaxM2 plate reader (1.0 µl in 200 µl total volume). 1.0 µl of ea IVT reaction was run on the Bioanalyzer 2100 (Agilent) for size confirmation and quantification. IVT reactions were then stored at -80˚C.

Quality control (QC).
Only PCR products that met standards for product size and homogeneity (as evaluated using Bioanalyzer analysis) were used to generate riboprobes. PCR products were expected to be within 100 bp of the correct anticipated size and to be represented by a single product.

QC standards for IVT products were also established. IVT products that are shorter than their predicted size were not used for ISH. However, IVT products frequently appear slightly larger than their predicted molecular weight, or as multiple peaks, due to secondary RNA structure. IVT products with multiple bands were not used for ISH unless the additional bands were determined to result from secondary structure.

Examples of typical Bioanalyzer electropherograms used in QC are shown in Figures 3 and 4.

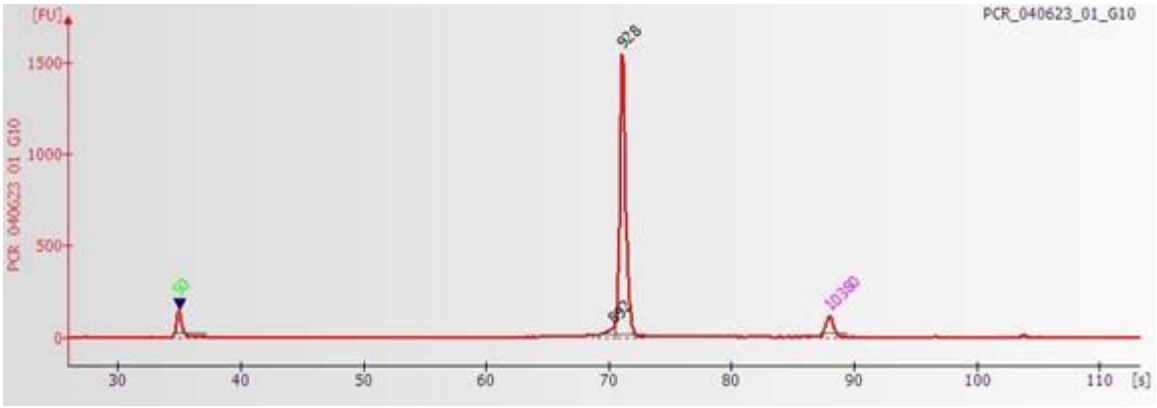

Figure 3. PCR product from *Neddl* gene, PCR_040623_01_G10 (size of peak, 928 bp), shown as an electropherogram from the Bioanalyzer.

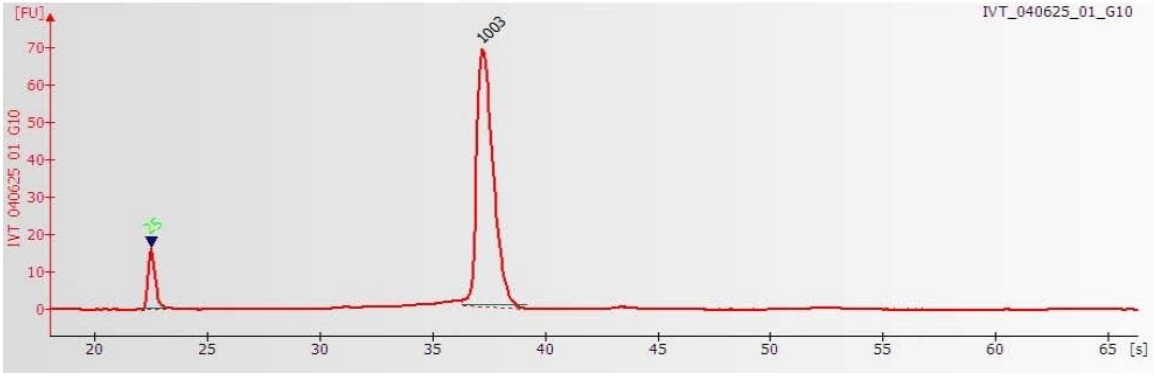

Figure 4. IVT reaction product from *Neddl* gene, IVT_040625_01_G10 (size of peak, 1003 bp).

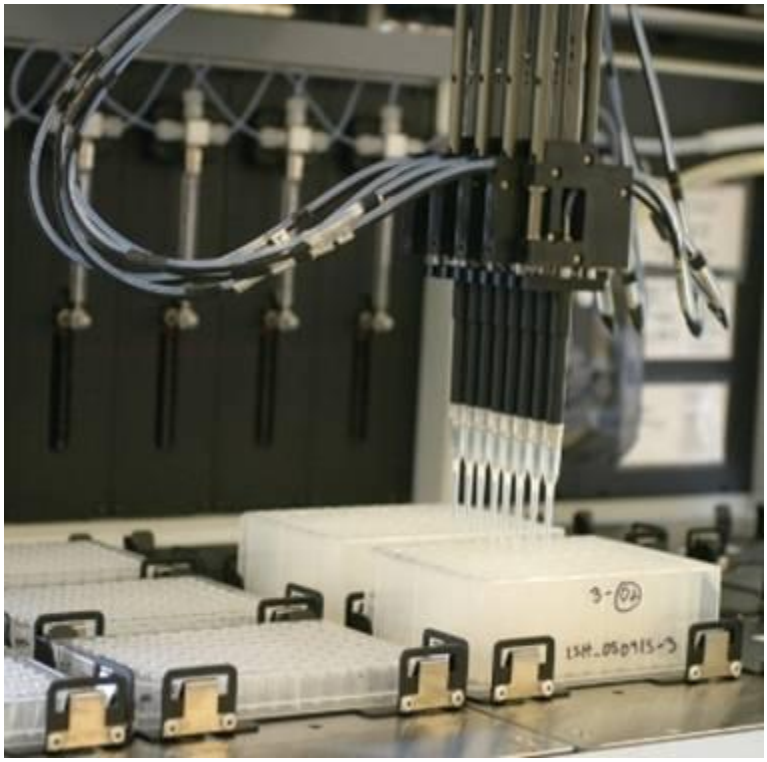

Figure 5. A Perkin Elmer Multiprobe II instrument was used for initial normalization of IVT reaction products to 30 ng/µl with THE, aliquotting hybridization mix at 400 µl per well to ISH probe plates and final addition of 4 µl (30 ng/µl) probe to ISH probe plates.

Dilutions

IVT reactions were diluted to working stocks of 30 ng/µl with THE (0.1 mM sodium citrate pH 6.4, Ambion). IVT reactions were stored in aliquots of approximately 36 wells/plate in one or two use volumes to minimize freeze/thaw cycles. Diluted IVT reactions were stored at -80˚C.

For hybridization, the probe was diluted 1:100 (to 300 ng/ml) into *in situ* hybridization buffer (Ambion) in 96-well ISH probe plates. Each well provides the probe for one ISH slide. Probe plates were stored at -20˚C until use.

TISSUE PREPARATION

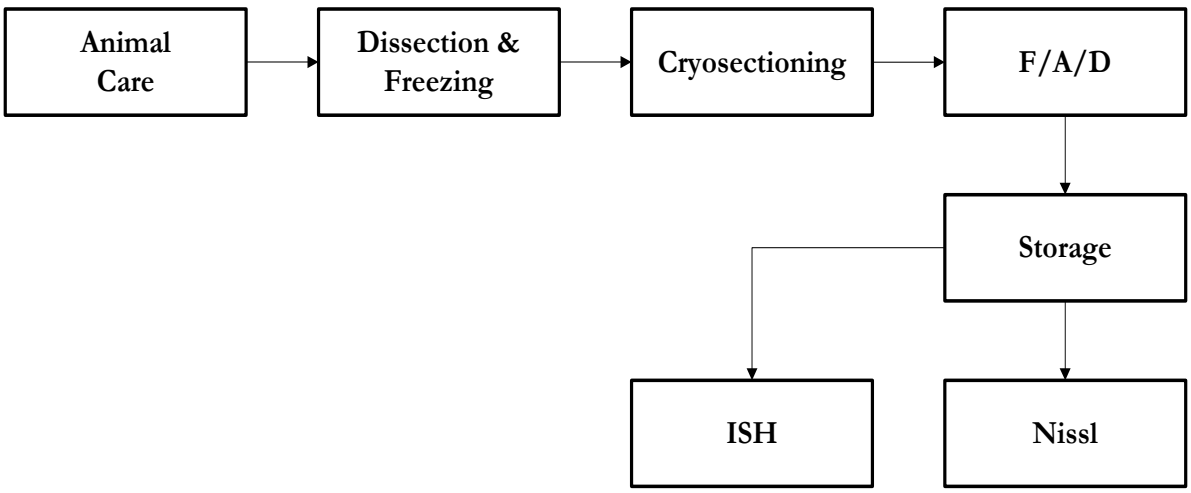

Figure 6. Allen Institute Tissue Preparation Workflow. (F/A/D refers to fixation, acetylation, and dehydration).

Animal care

The Atlas used 8 week (56 day) old adult C57BL/6J male mice. In order to maintain a consistent genetic stock, mice were purchased from The Jackson Laboratory West. Mice were acclimated to the facility for at least 4 days prior to sacrifice. Mice were group-housed (5 per cage) in micro ventilated cages with quarter inch bed-o-cobs bedding and igloos for environmental enrichment.

Dissection and freezing

Standard procedures were developed to isolate, cut, fix and pre-treat tissue to preserve macro and cellular morphology and to produce the best signal to noise ratio for ISH. Mice were transferred from the vivarium to the procedures room with efforts to minimize stress during transfer. If mouse body weight falls outside of the normal range (18.8 to 26.4 g), the brain was not used in the ISH process. Mice were anesthetized with 0.5% isoflurane. A grid-lined freezing chamber was designed to allow for standardized placement of the brain within the block in order to minimize variation in sectioning plane. Chilled OCT was placed in the chamber, and a thin layer of OCT was frozen along the bottom by brief placement of the chamber in a dry ice ethanol bath. The brain was rapidly dissected and placed into the OCT. The orientation of the brain was adjusted using a dissecting scope, and the freezing chamber containing OCT and brain were frozen in a dry ice/ethanol bath. Brains were stored at -80˚C.

Cryosectioning

The fresh frozen brains were sectioned at 25 µm on Leica 3050 S cryostats. This thickness was optimal for minimizing sectioning artifacts, and was adequate for probe penetration into the section during the ISH procedure. Each OCT block containing a fresh frozen brain was trimmed in the cryostat until reaching the desired starting section.

One brain sectioned in sagittal plane was typically used to generate 8 series of 5 slides each (Figure 8), each containing four 25 µm thick sections. One coronal-sectioned brain will generate 8 series of 15 slides (Figure 9). Slides were grouped into series (8 series per brain) that contain sections 200 µm apart, allowing for uniform sampling every 200 µm across the entire brain for each gene. A given series was either hybridized to a single gene or used for Nissl staining for anatomical reference. Typically, 6 series per brain were used for ISH, and 2 series were used for Nissl. Occasionally datasets were generated with higher density (100 µm) sampling per gene.

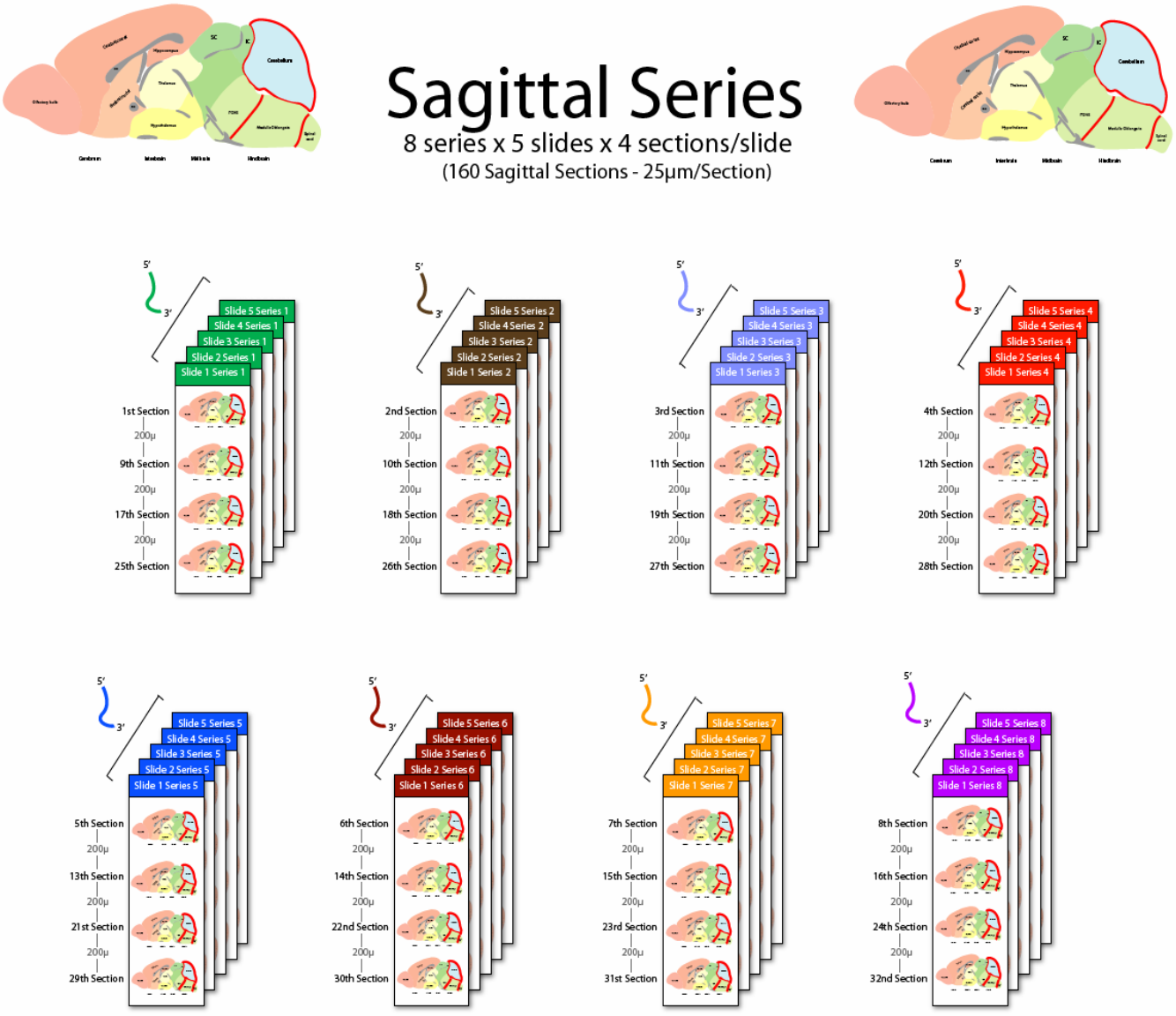

Figure 8. Standard series schema for a brain sectioned in sagittal plane.

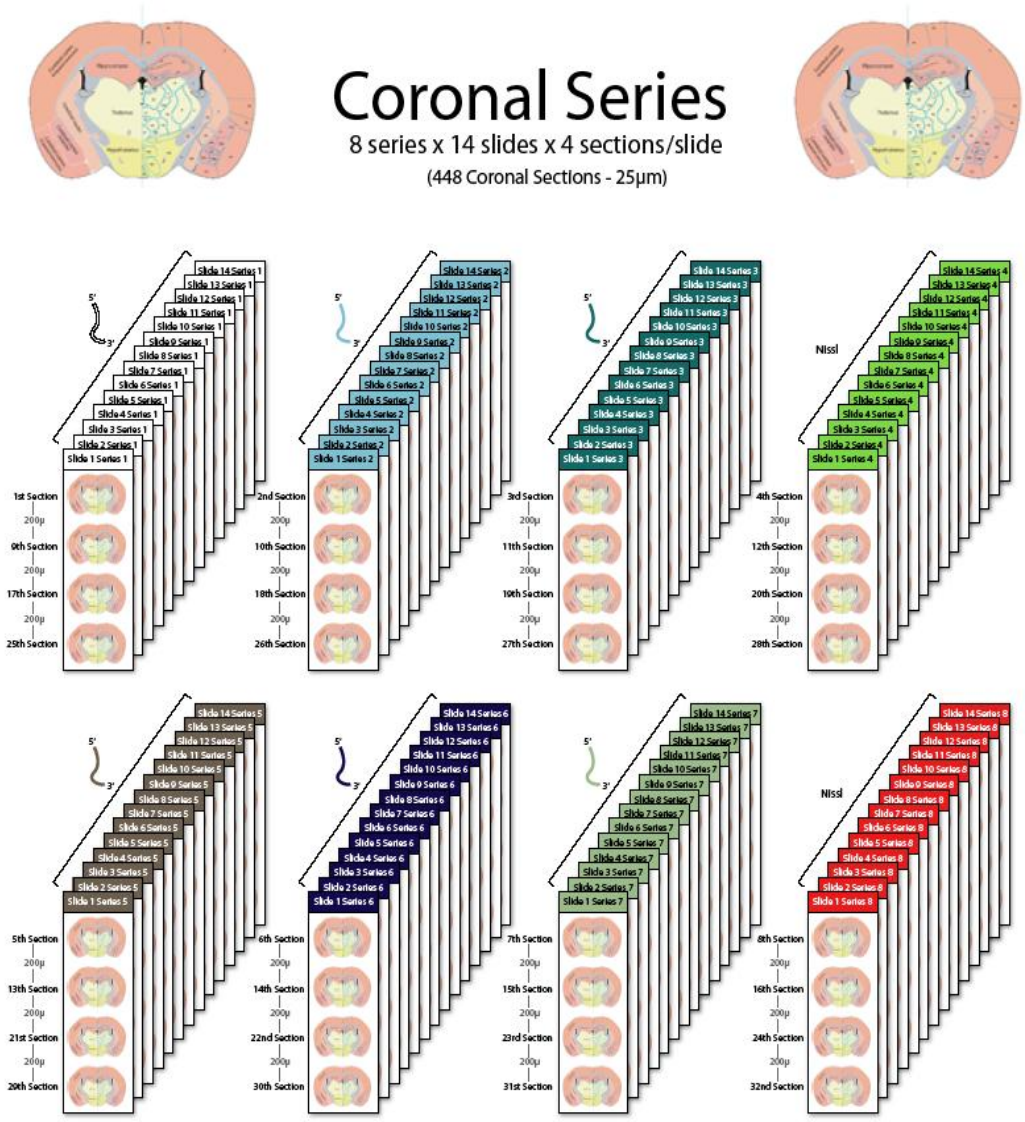

Figure 9. Standard series schema for a brain sectioned in coronal plane.

Fixation, acetylation and dehydration (F/A/D).

After allowing the tissue sections to air dry on the slides for a minimum of 30 minutes, the tissue was fixed in 4% neutral buffered paraformaldehyde (PFA) for 20 minutes and rinsed for 3 minutes in 1x PBS. Acetylation is necessary to reduce non-specific probe binding to the tissue sections. Several chemical functional groups in proteins, such as amine and carboxylate groups, are believed to be induced by nonspecific probe binding, consequently leading to higher background levels and lower signal/noise ratios. Acetylation of positively charged amine groups by treating tissue sections with acetic anhydride reduced nonspecific binding of negatively charged nucleic acid probes. For acetylation, the tissue was equilibrated briefly in 0.1 M triethanolamine with 0.25% acetic anhydride. Immediately following acetylation, the tissue was dehydrated through a graded series containing 50%, 70%, 95%, and 100% ethanol.

Following the dehydration process, each slide is analyzed microscopically to ensure section quality. Slides that pass QC were stored at room temperature in Parafilm-sealed slide boxes.

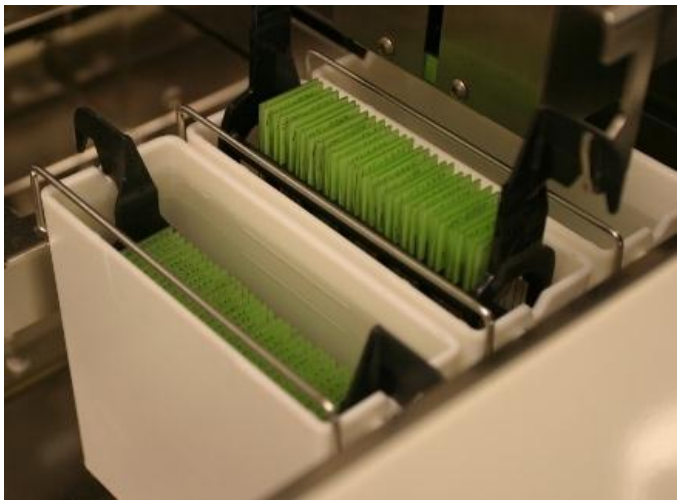

Figure 10. Leica Autostainer XL was used for the fixation and dehydration tasks. Acetylation is performed manually.

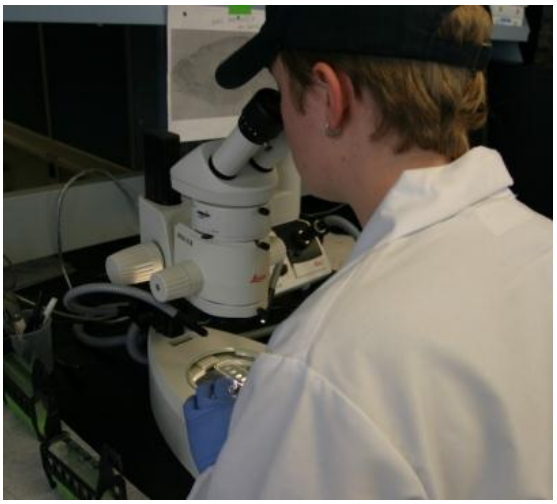

Figure 11. Section quality is confirmed following F/A/D.

Reagent preparation

In accordance with good laboratory practices (GLP), the Allen Institute has implemented a comprehensive reagent tracking system. This includes a detailed document control process to support the preparation of each reagent. Additionally, the Allen Institute has developed custom reagent preparation laboratory notebooks to facilitate the unique requirements of our processes. A complete list of purchased and prepared reagents used during the ISH process is provided in Appendix 2.

In situ hybridization (ISH)

In situ hybridization was used to detect specific RNA sequences within a section of tissue. The Atlas used a non-radioactive, digoxigenin (DIG) based technique to label cells expressing a particular transcript.

Slides were assembled into flow-through chambers using small spacers, a backplate, and clips, and then were placed into a temperature-controlled rack and positioned on a Tecan Genesis liquid handling platform. All solutions were added using a computer-controlled liquid handling system. Temperature of the rack and solutions was controlled by water circulator baths that are regulated by the same computer. All steps were performed at room temperature unless otherwise indicated. All solutions used in steps up to and including hybridization were made with DEPC-treated water in sterile plastic vials or glassware baked at 180˚C. Several solutions were degassed in order to prevent the formation of bubbles in the hybridization chamber. See Appendices I and II for the full details of the Allen Institute *in situ* hybridization protocol.

Prior to hybridization, the tissue was treated with hydrogen peroxide to block endogenous peroxidase activity, and was treated with proteinase K to increase the permeability of the tissue, allowing penetration and hybridization of the labeled probe to its complementary target mRNA. The tissue was incubated with the digoxigenin-labeled riboprobe for 5.5 hours at 63.5˚C. Once the hybridization process was complete, the tissue was treated with a sequence of increasingly stringent washes containing decreasing salt concentrations.

Detection of the bound probe was a multi-step procedure. First, a succession of blocking steps inhibited endogenous protein activity from interfering with the colorimetric enzymatic reactions. The colorimetric

reaction itself was a four part process, starting with the addition of a horseradish peroxidase (HRP)-conjugated anti-digoxigenin antibody. A Tyramide Signal Amplification (TSA) step was utilized to maximize sensitivity. The tissue was incubated with a biotin-coupled tyramide. Tyramide was converted by HRP into a highly reactive oxidized intermediate which binds rapidly and covalently to cell-associated proteins at or near the HRP-linked probe. This results in significant (up to a hundred-fold) amplification of bound biotin molecules available for detection. These biotin molecules were then bound to neutravidin-alkaline phosphatase (AP). A colorimetric reaction occurred when the alkaline phosphatase conjugated to the neutravidin enzymatically cleaves the phosphate from 5-bromo-4-chloro-3-indolylphosphate (BCIP), and two of the resulting indoles reacted with nitroblue tetrazolium (NBT) to produce a blue particulate precipitate at the sites of probe binding. Finally, the tissue was treated with a wash buffer containing EDTA followed by fixation with 4% PFA. These steps halted the colorimetric reaction.

Each ISH run contained two controls. The positive control was hybridized with antisense *Drd1a* probe, a probe which provides strong staining in the striatum and cortical layer 6b. The negative control consisted of a slide hybridized with no probe. As part of the quality control process, these slides were analyzed visually after the ISH run was complete. The positive control slide was assessed for overall signal quality and intensity, and the negative control slide was assessed for background staining (see *ISH Platform Controls*). Significant deviations from the expected expression resulted in failure of the entire run.

The Allen Mouse Brain Atlas *in situ* hybridization platform used a Tecan robot with GenePaint technology developed by Dr. Gregor Eichele's laboratory at the Max Planck Institute and Baylor College of Medicine (www.genepaint.org). The ISH protocol used for the Atlas and executed on the Tecan platform is detailed in Appendix 1.

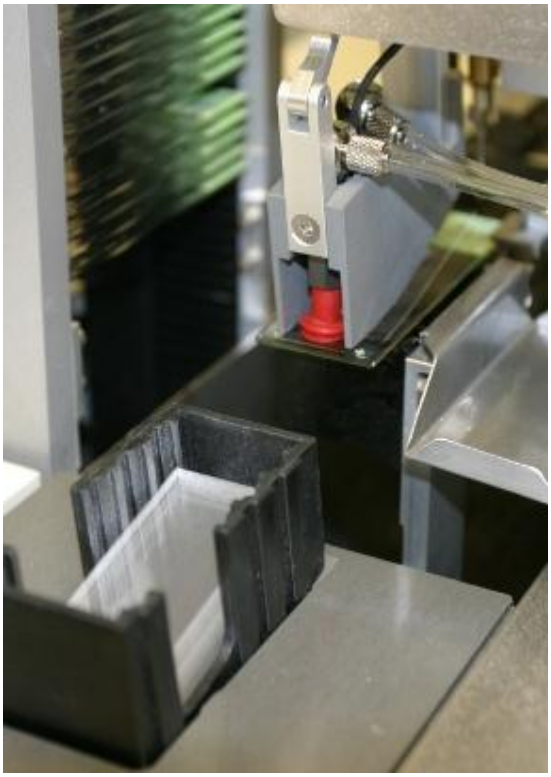

Figure 12. Customized Tecan robots were used to pipette solutions onto slides at programmed intervals, allowing the majority of the ISH procedure to be performed in an automated fashion (left). The "gripper" of a Leica CV5030 instrument automatically applied a coverslip to specimen slides (right).

Coverslipping and slide cleaning process

Immediately following the ISH process, slides were prepared for coverslipping. The slides were disassembled from the Tecan flow-through chambers, washed four times (one minute per wash) in 70% ethanol at pH 2.1, immediately followed by four one-minute rinses in MilliQ water. The wash steps were performed on a Leica Autostainer XL. The slides were then coverslipped with 22 x 55 mm cover slips with Hydro-Matrix Water

Solved Mounting Medium on a Leica CV5030 Coverslipper. Coverslipped slides were incubated overnight at 37˚C to solidify the mounting media. Prior to scanning, the slides were cleaned to remove all excess mounting media and other debris and dust that could interfere with the scanning process.

NISSL STAINING

Nissl staining is a histological procedure that labels Nissl bodies, the ribosomal RNA associated with the rough endoplasmic reticulum. A series of Nissl slides were generated for each brain to serve as a cytoarchitectural reference for the ISH data.

After a brain was sectioned, series 4 and 8 were baked at 37 ˚C for 1-5 days, and stored in dessicated containers until staining. Sections were defatted with xylene or the xylene substitute Formula 83, and hydrated through a graded series containing 100%, 95%, 70%, and 50% ethanol. After incubation in water, the sections were stained in 0.25% thionin, which stained the Nissl bodies purple-blue. Next, the sections were differentiated and dehydrated in water and a graded series containing 50%, 70%, 95%, and 100% ethanol. Finally, the slides were incubated in xylene or Formula 83, and coverslipped with the mounting agent DPX. After drying, the slides were analyzed under a microscope to ensure staining quality. Slides that have passed QC were stored at room temperature in slide boxes before being cleaned and scanned.

IMAGE CAPTURE

Two image acquisition platforms were used for scanning and digitizing slide image data.

Image capture system

Initially, images were generated using a high-throughput automated microscopy platform developed at the Allen Institute for Brain Science. Each stand-alone image capture system (ICS) unit consisted of a Leica DM6000B automated brightfield microscope, Leica DC500 camera, Ludl BioPrecision stage with automated slide loader, Microscan CCD-3 barcode imager, and an HP wx6200 dual-processor workstation. Custom ScopeController software combined with ImagePro Plus commercial software handled all system integration, configuration and image acquisition processes. Following transfer from 25 slide cassette holders to a microscope stage, each slide was barcode scanned for data tracking purposes, and a color, white balance, and background correction check was performed. For image acquisition, each slide was initially prescanned with a 1.25x objective, a composite image was made, and a thresholding method was used to locate the tissue section on each slide. A bounding box was calculated to encompass each tissue section. After switching to the 10x objective, two points were automatically selected for each tissue section, and these points were used to calculate the focal plane for the tissue. Finally, each section was scanned at 10x, with numerous tiles captured per section. These tiles were later stitched by a custom algorithm into a full image per section. Scanning on the ICS typically required 15-20 minutes per slide. The image resolution of this system was 1.05 µm per pixel.

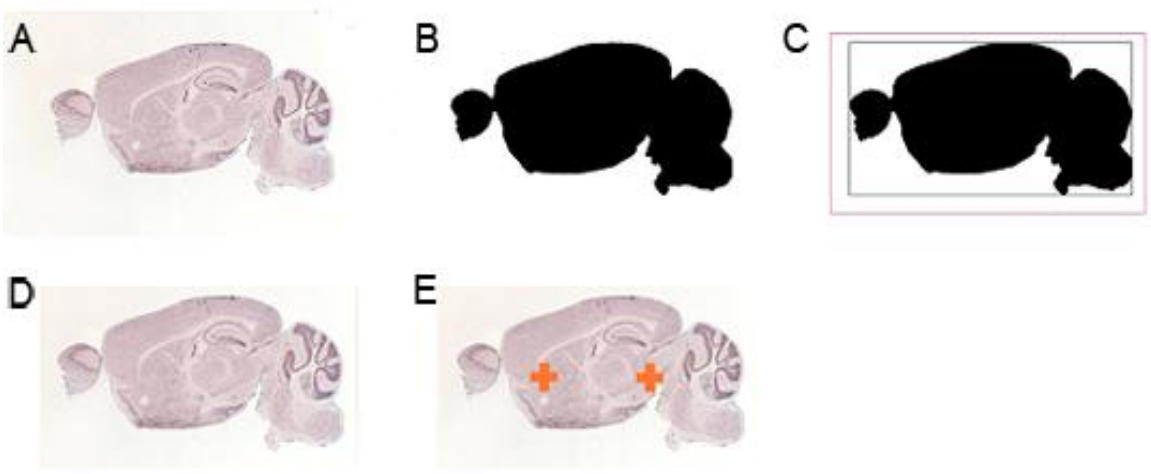

Figure 15. ICS tissue identification and focusing points.

A: Four 1.25 tiles stitched together to make a composite image of a section. B: Thresholding to locate tissue section on the slide. C: Bounding box that encompasses the section. D: Region on the slide to be scanned. E: Two focus point selected across the section to determine the focal plane across the tissue.

Aperio ScanScope

The current platform used for scanning slides for the Atlas is the ScanScope (Aperio Technologies, Inc; Vista, CA). The line scan camera continually adjusted for focus based on a variable number of focus points. The scanner used a 20x objective to create a full slide image that was downsampled in software to minimize data volume acquired for the project. The downsampling provided similar image resolution (1.00 µm/pixel) to the ICS scanning systems.

Quality control (QC) of ISH slides and captured images

There were multiple quality control steps throughout the process. After imaging, there were two main QC checkpoints for the data. First, there is a QC step to ensure that image quality is adequate. All images were visually inspected in the Laboratory Information Management System (LIMS). Individuals trained to perform this QC step decided whether to accept or reject each image. In general, images were examined for focus, lighting, artifacts (including bubbles and debris), and overall tissue quality. Some image failures could be recovered by either rescanning the slide, or re-coverslipping and rescanning the slide. After the image- and slide-based QC step, there was a final QC step in which the data analysis team ensured that all passed images presented to the public meet an acceptable standard for consistency. Metrics for QC include anatomic normalcy, dissection quality, section orientation, signal-to-noise ratio, and in some cases, consistency with published literature or other evidence for expression.

SubImages for 80347982

Export Search

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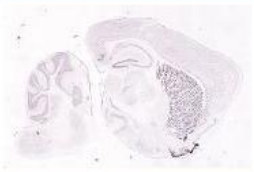
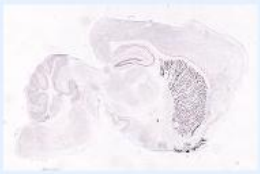
0500010706	0010	BRAIN		0500010706_2_zoom	image not available	image not available	☑	☐
0500010706	0016	BRAIN		0500010706_3_zoom	image not available	image not available	☑	☑
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Figure 16. LIMS view of section images for a slide during the QC process.
Note that the last two images are marked as failed for not meeting quality standards.

APPENDIX 1. ALLEN MOUSE BRAIN ATLAS *IN SITU* HYBRIDIZATION PROTOCOL.

Cycles	Time (min)	Volume (µL)	Reagent	Function	temp	Time (min)	~ Run Time (@ HR)	Manual notes
5	5 min	300	3% H_2O_2 in MeOH	Blocks peroxidase activity	24ºC	25		
7	5 min	300	PBS (1)	Washes, and restores pH	24ºC	35	1	
2	5 min	300	0.2M HCl	Permeates cell membrane	24ºC	10		
4	5 min	300	PBS (2)	Washes, and restores pH	24ºC	20		
1	5 min	400	PK(+) buffer	Equilibrates samples for Proteinase K addition	24ºC	5	1:35	
2	10 min	300	Proteinase K	Degrades proteins, allowing riboprobes to penetrate the tissue	24ºC	20		
6†	5 min	300	PBS (3)	Washes, and restores pH	24ºC	35	2:30	Add Hyb Mix
2	10 min	300	4% PFA (1)	Fixes tissue	24ºC	20	2:55	
7	5 min	300	PBS (4)	Washes, and restores pH	24ºC	35	3:25	
2	15 min	300	Hybe mix (1)	Stabilizes riboprobe	64ºC	30		Probes into 65° bath for 30 min.
1	15 min		heat to 64ºC	Incubation temperature	64ºC	15	4:10	
1		300	riboprobe addition	Targets nuclear mRNA	64ºC			Add O/N reagents
1	5.5 hrs			Hybridization	64ºC	330		
5	5 min	300	5 x SSC	Washes slides post hybridization	63.5ºC	25	10	
5	10 min	350	Formamide I	Reduces the amount of non-specific binding	63.5ºC	50		
5	12 min	350	Formamide II		63.5ºC	60	12:15	
4	8 min	300	0.1 x SSC	Washes slides post hybridization	63.5ºC	24		
1	8 min	300	0.1 x SSC		63.5ºC	8		
4	5 min	300	NTE	Washes and restores pH	24ºC	20		
3	5 min	300	20 mM iodoacetamide	Blocking reagent	24ºC	15		

3	5 min	300	20 mM iodoacetamide	Blocking reagent	24ºC	15	13:37	
4	5 min	300	NTE	Washes and restores pH	24ºC	20		
2	5 min	300	TNT	Washes and restores pH	24ºC	10		
3	5 min	300	4% lamb serum	Blocking reagent	24ºC	15		
3	5 min	300	4% lamb serum	Blocking reagent	24ºC	15		
4	5 min	200	TNT	Washes and restores pH	24ºC	20		
2	10 min	300	TNB blocking buffer	Equilibrates Samples	24ºC	20		
2	5 min	200	TNT	Washes and restores pH	24ºC	10	15:27	
2	5 min	300	maleate wash buffer	Washes and restores pH	24ºC	10		
2	10 min	350	blocking reagent	Blocking reagent	24ºC	20		
2	5 min	300	maleate wash buffer	Washes and restores pH	24ºC	10		
2	5 min	250	TNT	Washes and restores pH	24ºC	10		
3	5 min	350	TMN	Equilibrates tissue	24ºC	15		
4	5 min	200	TNT	Washes and restores pH	24ºC	20		
4	10 min	300	TNB blocking buffer	Equilibrates Samples	24ºC	40	17:22	
2	30 min	350	□DIG-POD	1˚ Antibody	24ºC	60		
6	5 min	250	TNT	Washes and restores pH	24ºC	30	18:52	
1	30 min	250	PE tyramide-biotin	Signal amplification reagent	24ºC	30	19:22	Add Neutravidin; Add 4% PFA back to deck
6	5 min	300	maleate wash buffer	Washes and restores pH	24ºC	30		
2	20 min	350	Neutravidin	Forms a conjugate with Biotin	24ºC	40		
6	5 min	300	maleate wash buffer	Washes and restores pH	24ºC	30	21:08	
4	5 min	250	TNT	Washes and restores pH	24ºC	20	21:28	
2	5 min	400	TMN	Equilibrates tissue prior to color reaction	24ºC	10		QC BCIP/NBT and add to deck
3‡	20 min	350	BCIP/NBT	Forms the color reaction	24ºC	60		

4	x	400	System liquid	Washes and stops color reaction	24ºC	10		
1	x	300	NTE	Washes and restores pH	24ºC	5		
1	10 min	250	4% PFA	Fixation step	24ºC	10		
3	x	400	System liquid	Final wash	24ºC	10	23:03	

† Changed from 7 cycles to 6 cycles.

‡ Changed from two 15 minute incubation and one 10 minute incubation (total 40 minutes) to three 20 min incubations (total 60 min)

APPENDIX 2. REAGENTS USED IN THE ISH PROCESS

Reagent	Role	Mode of Action	pH	Specs
3% H_2O_2 in MeOH	Blocks endogenous peroxidase/pseudoperoxidase activity	Tissue peroxidase/pseudoperoxidase will cause the TSA reagent to deposit biotin in regions where there is no probe binding, giving rise to false positive signal. Saturation of the enzyme with H_2O_2 reduces or eliminates this source of background.		10% H_2O_2 (Stock Conc. 30%) 90% Methanol
Phosphate Buffered Saline (PBS)	Washes and restores pH	Biologically neutral wash solution. Removes methanol/H_2O_2.	7.4	0.137M NaCl 0.0027M KCl 0.008M Sodium phosphate dibasic 0.002M potassium phosphate monobasic 0.0005% Tween 20
0.2 M HCl	Reduces background	Weak acid treatment dissociates histones from DNA, hydrolyzes tissue proteins.		
PBS	Washes and restores pH	Biologically neutral wash solution.	7.4	See previous description of this reagent.
PK Buffer	Equilibrates samples for Proteinase K addition	Tris-HCl buffers are used to control pH in the physiological range (~pH 7-8).	8	0.005M EDTA 0.05M Tris 0.0005% Tween 20
Proteinase K	Digests proteins, allowing riboprobes to penetrate the tissue	Proteinase K is a hemolytic serine protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids. It partially reverses the effects of tissue fixation with paraformaldehyde, removing masking proteins that hinder riboprobe binding and permeabilizing tissue.	8	0.005M EDTA 0.05M Tris 0.0175U Proteinase K/mL PK Buffer 0.0005% Tween 20
PBS	Washes and restores pH	Stops the enzymatic action and removes proteinase K from the section.	7.4	See previous description of this reagent.

4% Paraformaldehyde (PFA)	Fixation	Fixes tissue by linking the nitrogen of a lysine –R group to the nitrogen of a peptide bond by means of a –CH2-. This cross-linking serves to trap nucleic acids within the cells, yet allows free movement of smaller molecules	7.4	**Stock solution** 200g/L of 95% prilled paraformaldehyde 0.685M NaCl 0.0135M KCl 0.04M Dibasic Sodium phosphate 0.01M Monobasic potassium phosphate (Diluted to 4% working concentration in PBS)
PBS	Washes and restores pH		7.4	See previous description of this reagent.
In Situ Hybridization Buffer	Stabilizes riboprobe	Dextran Sulfate accelerates the rate of hybridization 10-fold. Denhardt's solution is a mixture of high-molecular weight polymers capable of saturating non-specific binding sites.		Purchased commercially (Ambion). Cocktail of Dithiothreitol (DTT), Dextran Sulfate, Denhardt's solution and Formamide.
Hybridization solution with riboprobe	Binds specific cellular mRNA	Antisense riboprobes containing digoxigenin (DIG) conjugated UTP. Binds to cellular (sense) mRNA transcripts.		300 ng digoxigenin labeled riboprobe/ml hybridization buffer
5x SSC	Washes slides post hybridization to remove hybridization solution	Concentrated saline sodium citrate (SSC) solution stabilizes nucleic acid duplexes while washing away unbound probe.	7	0.75M NaCl 0.075M Sodium citrate 0.0005% Tween 20
Formamide I	Reduces non-specific binding of riboprobe	Decreases the melting temperature (T_m) of RNA/RNA hybrids, resulting in lower affinity of nonspecific binding RNA sequences.	7	0.6M NaCl 0.06M Sodium citrate 0.001% Tween 20 50% Deionized Formamide
Formamide II		Same as Formamide I, with a lower concentration of SSC.	7	0.3M NaCl 0.03M Sodium citrate 0.001% Tween 20 50% Deionized Formamide
0.1x SSC	Washes to reduce non-specific binding	Nucleic acid duplexes are less stable at low salt concentrations. Low [SSC] washes act to denature binding of less than perfect match RNA/RNA hybrids.	7	0.015M NaCl 0.0015M Sodium citrate 0.0005% Tween 20

NTE (Sodium Tris EDTA Buffer)	Washes and restores pH	Buffers pH, chelates divalent cations.	8	0.5M NaCl 0.01M Tris- (Hydroxymethyl)aminomethane 0.005M EDTA 0.0005% Tween 20
20mM iodoacetamide	Blocking reagent	Reacts with disulphide bridges and sulphydryl groups to reduce non-specific antibody binding.	8	0.5M NaCl 0.01M Tris-(Hydroxymethyl)aminomethane 0.005M EDTA 0.0005% Tween 20 20mM iodoacetamide
NTE	Washes and restores pH	Buffers pH, chelates divalent cations.	8	See previous description of this reagent.
TNT (Sodium Tris Buffer)	Washes and restores pH		7.6	0.1M Tris (Hydroxymethyl)aminomethane 0.15M NaCl 0.00075% Tween 20
4% Sheep Serum	Blocking reagent	Prevents non-specific binding antibodies.	7.6	0.1M Tris (Hydroxymethyl)aminomethane 0.15M NaCl 0.00075% Tween 20 4% Sheep Serum
TNT	Washes and restores pH		7.6	See previous description of this reagent.
TNB Blocking Buffer	Blocking reagent	Lowers nonspecific binding of the antibody by blocking general protein binding sites.	7.6	0.1M Tris (Hydroxymethyl)aminomethane 0.15M NaCl 0.005g NEN Blocking Buffer/mL TN 0.0005% Tween 20
TNT	Washes and restores pH		7.6	See previous description of this reagent.
Maleate Wash Buffer	Washes and restores pH	Sets pH to optimize binding of biotin-avidin.	7.5	0.09M Maleic Acid 0.1M NaCl 0.0005% Tween 20 pH with 0.175M NaOH

Blocking Reagent.	Blocking reagent	Sets optimal pH and lowers nonspecific binding of the antibody by blocking general protein binding sites.	7.5	0.09M Maleic Acid 0.1M NaCl 0.0005% Tween 20 pH with 0.175M NaOH 0.01g Roche Blocking Reagent/mL 1X MWB
Maleate Wash Buffer	Washes and restores pH		7.5	See previous description of this reagent.
TNT	Washes and restores pH		7.6	See previous description of this reagent.
TMN	Washes and sets pH	Sets pH in range that maximizes rate of alkaline phosphatase activity.	9.5	0.1M Tris (Hydroxymethyl)aminomethane 0.05M MgCl 0.5M NaCl 0.0005% Tween 20 2mM (-)-Tetramisole hydrochloride
TNT	Washes and restores pH		7.6	See previous description of this reagent.
TNB Blocking Buffer	Blocking reagent	Sets optimal pH and lowers nonspecific binding of the antibody by blocking general protein binding sites.	7.6	See previous description of this reagent.
Anti Digoxigenin antibody conjugated with horseradish peroxidase (HRP)	Primary antibody	The anti-DIG antibody is specifically directed against the DIG epitope incorporated into the riboprobe. Antibody fragments are used as they penetrate tissue easily, and show less non-specific binding than intact antibodies.	7.6	0.1M Tris (Hydroxymethyl)aminomethane 0.15M NaCl 0.005g NEN Blocking Buffer/mL TN 0.0005% Tween 20 0.25 U anti-DIG-HRP/mL TNB
TNT	Washes and restores pH	Removes unbound antibody.	7.6	See previous description of this reagent.
Tyramide Signal Amplification reagent	Signal amplification	Tyramide is converted by HRP into a highly reactive oxidized intermediate which binds rapidly and covalently to cell-associated proteins at or near the HRP-linked probe.		Tyramide conjugated to biotin; purchased commercially (Perkin Elmer).
Maleate Wash Buffer	Washes and restores pH		7.5	See previous description of this reagent.

Neutravidin conjugated to alkaline phosphatase (AP)	Binds to Biotin	Neutravidin binds to the bound biotin.	7.5	0.09M Maleic Acid 0.1M NaCl 0.0005% Tween 20 pH with 0.175M NaOH 0.01g Roche Blocking Reagent/mL 1X MWB 2.072 U of AP activity/mL buffer
Maleate Wash Buffer	Washes and restores pH		7.5	See previous description of this reagent.
TNT	Washes and restores pH		7.6	See previous description of this reagent.
TMN	Washes and sets pH	Sets pH in range that maximizes rate of alkaline phosphatase activity.	9.5	0.1M Tris (Hydroxymethyl)aminomethane 0.05M MgCl 0.5M NaCl 0.0005% Tween 20 2mM (-)-Tetramisole hydrochloride
BCIP/NBT with levamisole	Forms the color reaction	Alkaline phosphatase enzymatically cleaves the phosphate from BCIP, the resulting indoles undergo a redox reaction with NBT to produce a blue precipitate at the sites of probe binding. Levamisole inhibits endogenous alkaline phosphatase activity, which may produce a false positive signal.	9.5	0.1M Tris (Hydroxymethyl)aminomethane 0.05M MgCl 0.5M NaCl 0.0005% Tween 20 2mM (-)-Tetramisole hydrochloride 0.405mg/mL Nitroblue tetrazolium chloride 0.152mg/mL 5-Bromo-4-chloro-3-indoly-phosphate, 4-toluidine salt
Water	Rinse	Washes excess BCIP/NBT from slides.		
NTE (Sodium Tris EDTA Buffer)	Washes, restores pH, and stops BCIP/NBT color reaction	EDTA chelates divalent cations, inhibiting alkaline phosphatase activity.	8	See previous description of this reagent.
4% PFA	Fixation step	Tissue fixation and termination of the colorimetric reaction	7.4	See previous description of this reagent.
Water	Final rinse			