ABA Data Production Processes

The processes and procedures used by the Allen Brain Atlas (ABA) project were designed based on the methods developed by Dr. Gregor Eichele's Laboratory at the Max Planck Institute and Baylor College of Medicine. Building on these efforts, the ABA constructed a state of the art facility for performing *in situ* hybridization (ISH) in a highly consistent, automated, industrialized fashion. The production laboratory was built with specifications that allowed the ABA project a full capacity production of approximately 1,000 slides/4000 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 five robotic *in situ* hybridization platforms daily.

The ABA 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 ABA 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 ABA Standard Operating Procedures (SOPs). These SOPs are revision-controlled and changes to these procedures are reviewed extensively prior to implementation.

The ABA is operating in a mode of continuous process and automation improvement. Improving data quality is an ongoing process involving designed process experiments and assessment of the resulting data. There are also several short and longer term engineering projects in development to increase data quality and throughput as well as decrease manual involvement. These include projects to automate and streamline the process of cutting and transferring brain sections to slides, currently the most manual, variable, and lowest throughput aspect of the ABA data production process.

The ABA production processes is summarized in the flow chart below. The remainder of the document discusses these steps in detail (up to the LIMS QC process). The full protocol for the *in situ* hybridization process can be found in Appendices I and II. In addition, there are a number of quality control checkpoints in our production process that are detailed in Appendix III.

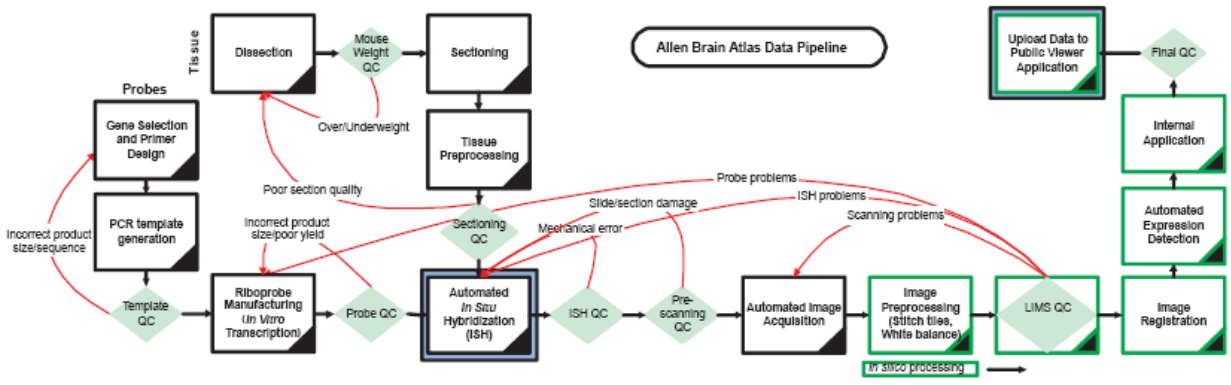

Figure 1 - *ABA production process*

Gene Selection and Riboprobe Synthesis

The ABA project is currently working to produce ISH data for 20,000 distinct mouse genes. The workflow and methods used for generating riboprobes are as follows:

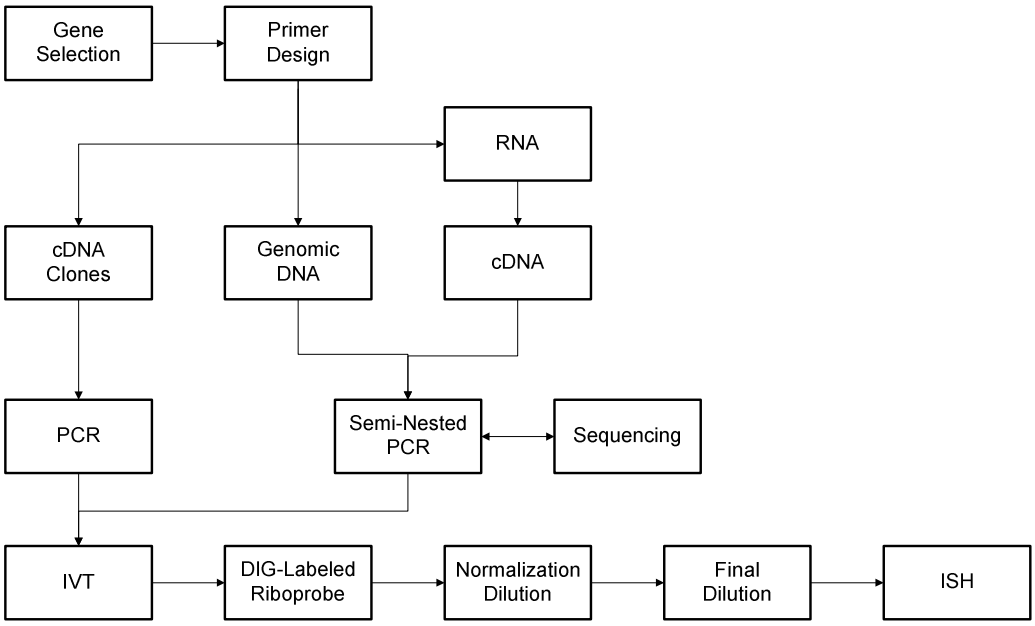

Figure 2 - *ABA Probe Production Workflow*

Gene Selection

The primary approach was to aim for 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 that were not represented in RefSeq.

Probe Design

We use a semi-automated process for probe design. Sequences are obtained from multiple sources including RefSeq, MGC, Celera, TIRG, Riken, and UniGene. One of three sources of DNA are used as templates for PCR; cDNA clones (MGC or Riken), pooled cDNA from mouse brain, or genomic tail DNA.

cDNA Clones

Clones are used as direct templates for PCR. Clones are stored as glycerol stock in 384-well and 96-well plates at -80˚C. Approximately 7,500 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. Consensus sequences with >98% homology across 80% of the total length are used to develop probes.

cDNA Templates

Pooled cDNA reactions made from mouse brain total RNA. When using mouse brain cDNA as a template probes are generated against sequences within a region 3000 bp from the 3' end. Approximately 9000 probes have been generated using cDNA as a PCR template.

Mouse brain cDNA preparation

Total RNA is isolated from homogenized wild type C57BL/6J mouse whole brain tissue using Ambion's ToTALLY RNA kit per the manufacturer's protocol. Total RNA is visualized on the Bioanalyzer and quantified by A_{260} readings using a SpectraMax-M2 plate reader (100ul at a 1:50 dilution). Typical yield is 120ug total RNA from one brain. Invitrogen's Superscript III RTS First-Strand cDNA Synthesis Kit is used for cDNA reactions. Reactions are performed in a 96 well plate as per manufacturer's protocol, using 5ug of Anchored oligo-dT-25. Each brain supplies enough RNA for 24x20ul reactions. cDNA reaction pools are made for each brain (480ul), which supplies template for 4x96 PCR reactions. 12 samples from each 96-well cDNA reaction plate are run on the Bioanalyzer for quality control.

Genomic DNA

Genomic DNA is isolated from mouse tail snips using Qiagen's DNAeasy Tissue Kit. Tail clips from two C57BL/6J mice (0.6 cm each) are combined for each DNA isolation reaction. DNA is run on a 0.8% agarose gel and visualized using SybrGreen and Amersham's Typhoon instrument. Size range isolated is 500bp-40kb. When using genomic DNA as a template probes are designed within exons with a minimal length of 400bp. Approximately 1000 probes have been generated using genomic DNA as a PCR template.

Primer Design

Gene-specific forward and reverse primers are designed according to the following sequence of events:

1. BLAST the clone sequences to find regions of homology in other genes/family members
2. Mask out repetitive and/or homologous sequences (described below)
3. Use Primer3 software for primer design using the following criteria:
4. Optimal size 18-20nt for clone templates, 22-24nt for cDNA and genomic DNA templates.
    4.1. GC content between 42-62%
    4.2. Product size between 300-1200nt (optimal > 600)
    4.3. Probe location within gene:
        4.3.1. No bias for clone templates.
        4.3.2. Within 3000bp of polyA tail for cDNA template.
        4.3.3. Within single exons for genomic DNA template.
5. Top primer pair (lowest penalty score) is chosen.
6. A nested reverse primer is also designed for cDNA and genomic DNA templates.
7. SP6 RNA polymerase binding sequence (GCGATTTAGGTGACACTATAG) is added to the reverse/nested primer as the antisense primer.
8. Primers are ordered from IDT in 96 well format and delivered at 10μM final concentration.

All gene sequences are blasted against the entire collection of transcript sequences described above. Regions of homology greater than 70% for regions over 100 bp are identified and are not used for probe design. (For a subset of genes in families with high homology these standards were relaxed to >90% for regions >120bp.) Within the remaining sequence primers are designed using Primer3 software (MIT). A nested approach is used for the generation of probes from mouse brain cDNA or genomic DNA are used as PCR templates. Three primers are generated; a forward, a reverse, and a nested primer. An initial PCR reaction is performed using the forward and reverse primers and the purified product is then used as the template for a second PCR using the same forward primer and a nested primer. When a cDNA clone is used as a template a single PCR is used therefore only a forward and reverse primer are used.

PCR

Standard PCR reactions are performed using Qiagen Taq Polymerase. All reactions are run in 96-well format for 35 cycles, 50μl total volume with final concentrations of 1.5mM $MgCl_2$ (1x Taq buffer), 0.5μm

oligonucleotide primers (IDT), 200µm dNTPs (Roche), and 1.25U Taq Polymerase. Clone glycerol stock, cDNA pool, or genomic DNA is used as template material (1.0 µl). A second round of PCR using the nested reverse primer is performed for cDNA and genomic DNA templates. PCR reactions are purified using Millipore's Montage 96 filter plate per the manufacturer's protocol, and eluted with 50µl of 10mM Tris pH 8.0 following a 30 minute room temperature incubation. PCR reactions are quantified by A_{260} readings using a SpectraMax-M2 plate reader (100µl at 1:25 dilution). Each PCR reaction is run on Agilent's Bioanalzyer 2100 (at 1:2 dilution) for product size confirmation and quantification. PCR reactions are stored at -20˚C.

Sequencing

All PCR products generated from cDNA and genomic DNA template are sequenced from both ends, using the forward primer and SP6. Sequencing is done on MegaBACE and ABI3700 capillary instruments at Rexagen.

***In Vitro* Transcription**

Standard *in vitro* transcription (IVT) reactions are performed using Roche's 10x DIG RNA Labeling Mix. All reactions are done in 96-well format for 2 hours at 37˚C, 30µl 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 (12µl, approx 600-1200ng) is used as template material. IVT reactions are purified using Millipore's Montage 96 filter plate per the manufacturer's protocol and eluted with 90µl of THE (0.1mM Sodium Citrate pH 6.4, Ambion) following a 30 minute room temperature incubation. IVT reactions are quantified using the RiboGreen HIGH assay (Molecular Probes) and the SpectraMax-M2 plate reader (1.0µl in 200µl total volume). 1.0 µl of each IVT reaction is run on Agilent's Bioanalyzer 2100 for size confirmation and quantification. IVT reactions are then stored at -80˚C.

Quality Control (QC)

PCR product size and homogeneity revealed by Bioanalyzer readings are important QC metrics during riboprobe production. PCR products that are not of the correct size (+/- 100bp) or that show multiple products are not used to generate riboprobes.

IVT products that are shorter than their predicted size are not used for ISH. 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 are not used for ISH unless the additional bands are determined to result from secondary structure.

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

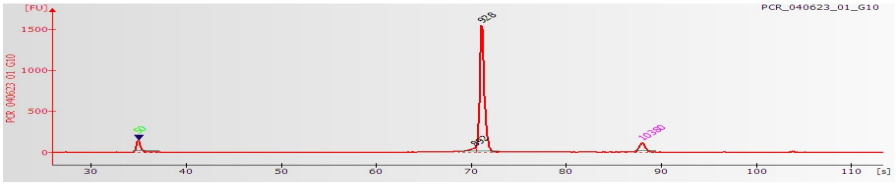

Figure 3 - *PCR product from Neddl gene, PCR_040623_01_G10 (size of peak shown in bp)*

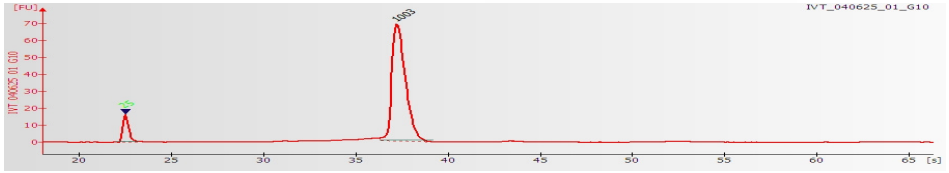

Figure 4 - *IVT reaction product from Neddl gene, IVT_040625_01_G10 (size of peak shown in bp)*

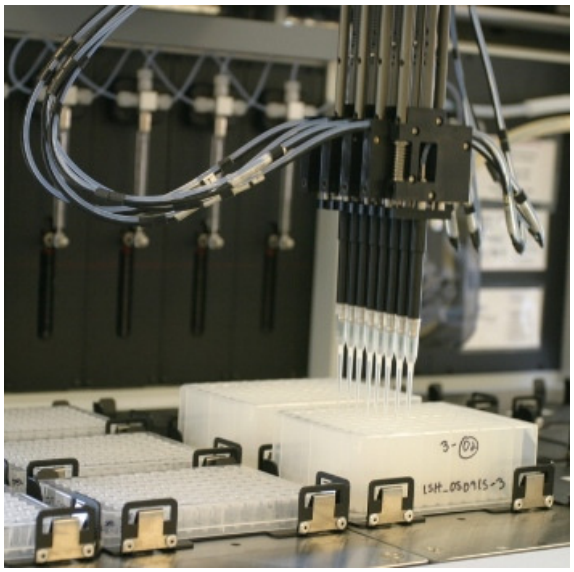

Figure 5 - *A Perkin Elmer Multiprobe II instrument is used for initial normalization of IVT reaction products to 30ng/ul with THE, aliquoting hybridization mix at 400ul per well to ISH probe plates and final addition of 4ul (30ng/ul) probe to ISH probe plates.*

Dilutions

IVT reactions are diluted to working stocks of 30ng/µl with THE (0.1mM Sodium Citrate pH 6.4, Ambion). Aliquots are stored in aliquots of 36 reactions/plate in one- or two-use volumes to minimize freeze/thaw cycles. IVT dilutions are stored at -80˚C.

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

Tissue Preparation

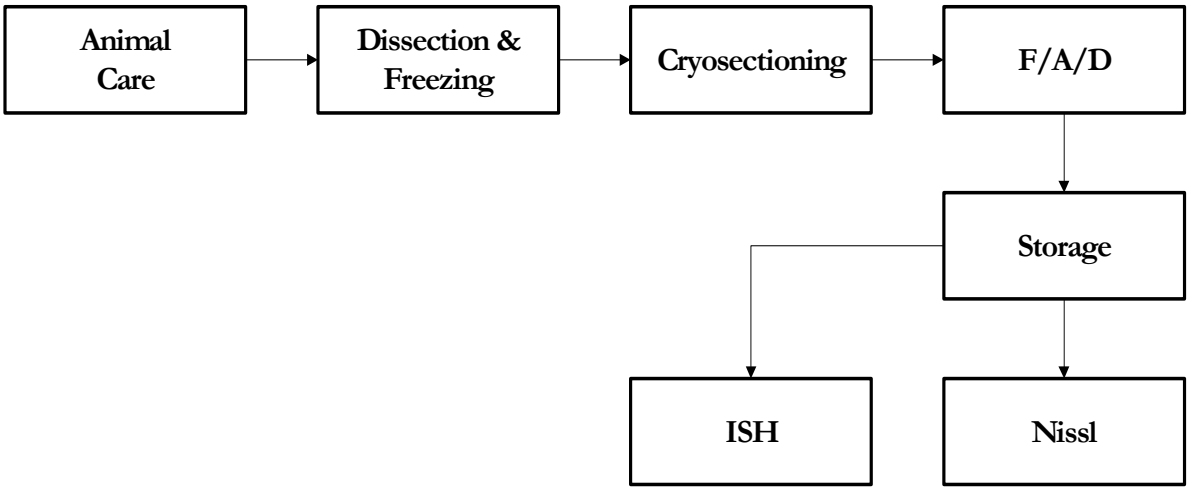

Figure 6 - *AIBS Tissue Preparation Workflow. (F/A/D refers to fixation, acetylation, and dehydration)*

Animal Care
8 week (56 day) old adult C57BL/6J male mice are used. In order to maintain a consistent genetic stock, mice are purchased from The Jackson Laboratory West. Mice are acclimated to our facility for at least 4 days prior to sacrifice. Mice are group housed (5 per cage) in micro ventilated cages with quarter inch bed-o-cobs bedding and igloos for environmental enrichment. They are maintained on a 12 h light/dark cycle with free access to water and Purina Lab diet 5001 mouse food.

Dissection & Freezing
Standard procedures were developed to isolate, cut, fix and pre-treat tissue to preserve as much macro and cellular morphology as possible and to produce the best signal to noise ratio for ISH. Mice are transferred from the vivarium to the histology core with efforts to minimize stress during the transfer. If mouse body weight falls outside of the normal range (18.8 to 26.4g), the brain is not used in the ABA ISH process. Mice are anesthetized with 0.5% isoflurane. Brains are rapidly dissected and frozen in OCT mounting medium in a grid-lined freezing chamber designed to allow for standardized placement of the brain within the block in order to minimize variation in sectioning plane. Brains are frozen at -80°C prior to sectioning.

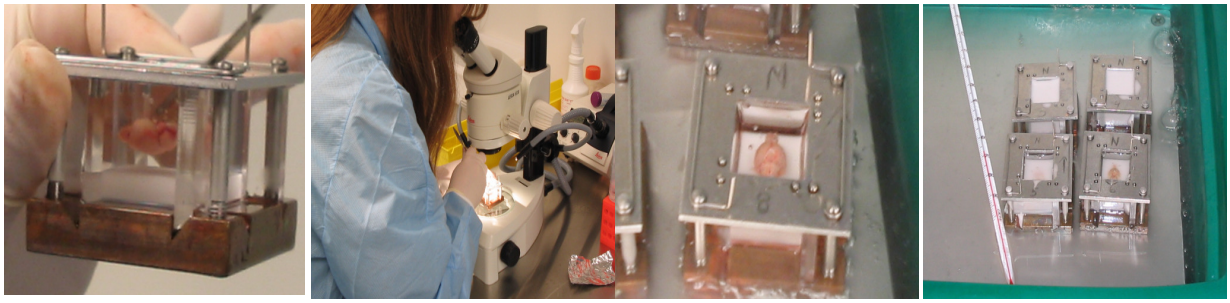

Figure 7 - *The brain is carefully removed and placed on aluminum foil on ice for approximately two minutes. A) A brain mold chamber is filled with Optimal Cutting Temperature (OCT) liquid and placed on an aluminum table above an alcohol bath containing dry ice. Once the bottom ¼" of OCT has solidified, the tissue is submerged into the chamber. B) A dissection microscope is used to align the tissue in the chamber. C, D) The chamber is placed back on the dry ice table for approximately 10 minutes to complete the OCT freezing process.*

Cryosectioning

The fresh frozen brains are sectioned at 25 µm on Leica 3050 S cryostats. This thickness is optimal for minimizing sectioning artifacts such as stretching and folding during cutting and adequate for probe penetration into the section during the ISH procedure. Each OCT block containing a fresh frozen brain is trimmed until reaching the plane of Figure 99 of the Atlas of the Mouse Brain for coronal sections, and to approximately Figure 128 for sagittal sections (*The Mouse Brain in Stereotaxic Coordinates*, Paxinos and Franklin, Second Edition, 2001). Starting at this plane of section, a fixed number of sections spanning the rest of the brain are collected.

One sagittal-sectioned brain will generate 8 series of 5 slides (Figure 8), each containing four 25 µm thick sections. One coronal-sectioned brain will generate 8 series of 15 slides (Figure 9). Slides are grouped into series (8 series per brain, with 4 sections per slide) that contain sections 200 µm apart, allowing for uniform sampling every 200 µm across the entire brain for each gene. All but the smallest neuro-anatomical structures should be represented in at least one section at this sampling density. A given series is either hybridized to a single gene or used for Nissl staining for anatomical reference.

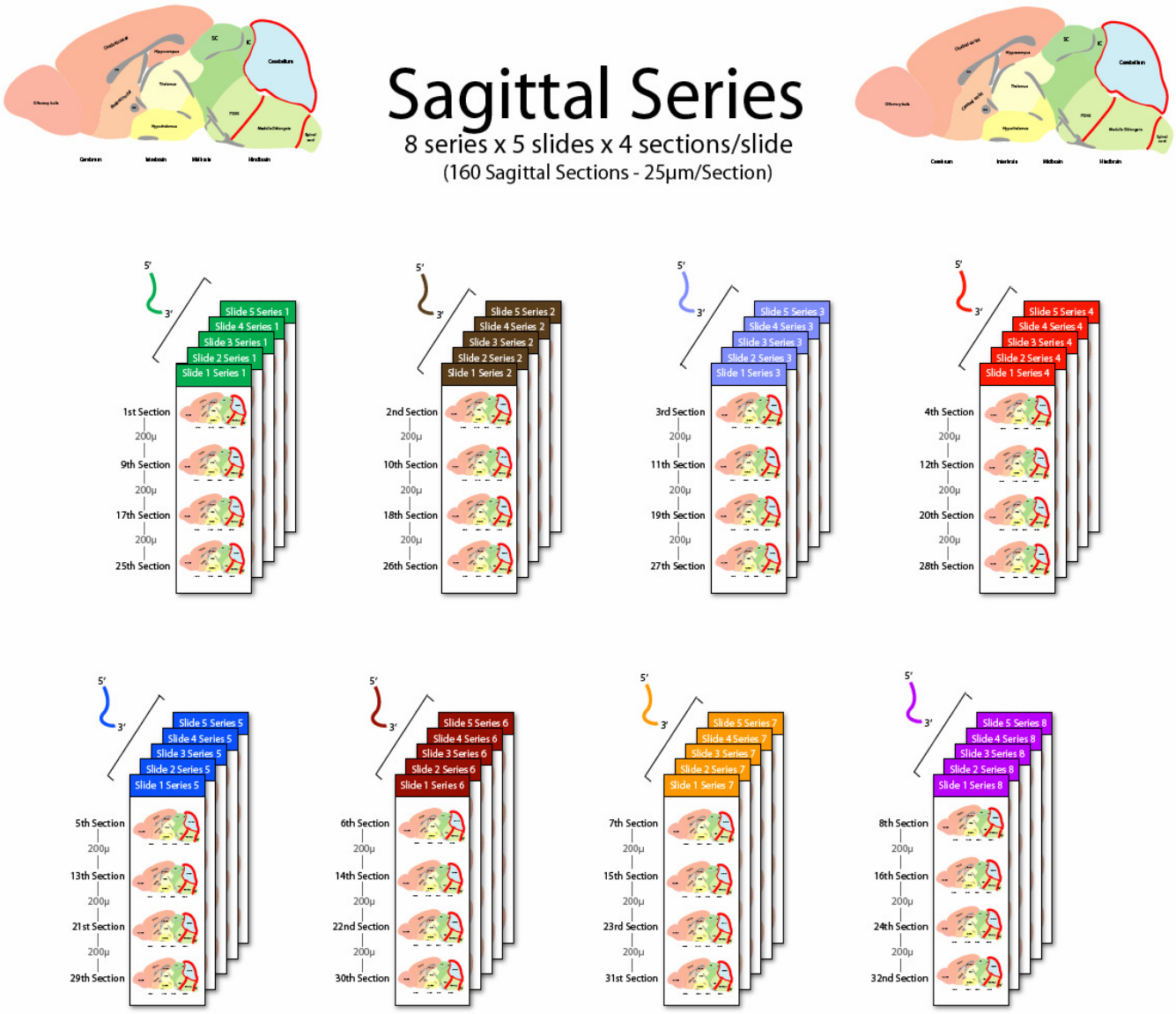

Figure 8 – *Standard series schema for a sagittally-sectioned brain*

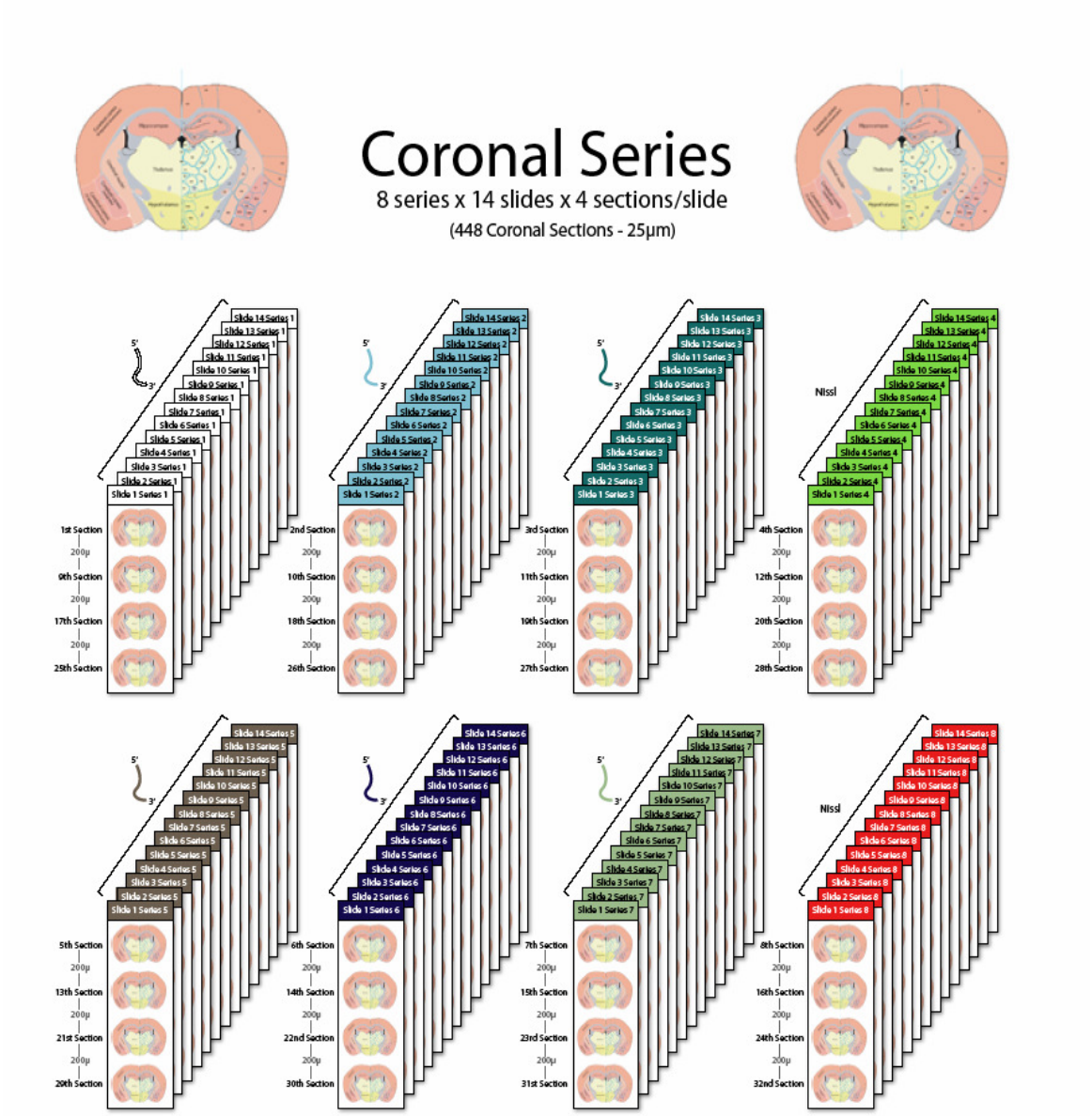

Figure 9 - *Standard series schema for a coronally-sectioned brain*

Fixation, Acetylation and Dehydration (F/A/D)

After allowing the sections to air dry on the slides for a minimum of 30 minutes, the tissue is fixed in 4% neutral buffered paraformaldehyde (PFA) for 20 minutes and rinsed for 3 minutes in 1x PBS. Next, the tissue is equilibrated briefly in 0.1 M triethanolamine and acetylated for 10 minutes in 0.1 M triethanolamine with 0.25% acetic anhydride. 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 induce 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 reduces nonspecific binding of negatively charged nucleic acid probes. Immediately following acetylation, the tissue is 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 are stored at room temperature in Parafilm-sealed slide boxes.

Figure 10 - *Leica Autostainer XL's are used for the fixation and dehydration tasks. Acetylation is performed manually.*

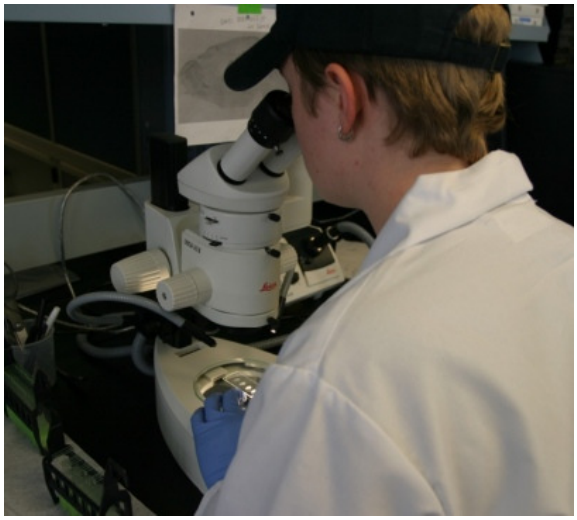

Figure 11 - *Section quality is confirmed following F/A/D.*

Reagent Preparation

The Allen Institute has an established reagent preparation department which supports the full spectrum of processes associated with our ISH Platform. 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 II.

In situ Hybridization (ISH)

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

Slides are integrated into flow-through chambers which are then placed into a temperature-controlled rack and positioned into a Tecan Genesis liquid handling platform. All solutions are added using a computer-controlled liquid handling system. Temperature of the rack and solutions is controlled by water circulator baths that are regulated by the PC that controls the liquid handling system. All steps are performed at room temperature unless otherwise indicated. All solutions used in steps up to and including hybridization are made with DEPC-treated water in sterile plastic vials or glassware baked at 180°C. Several solutions are 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 fixed, acetylated, and dehydrated tissue undergoes steps designed to block endogenous peroxidase activity and to increase the permeability of the tissue, allowing penetration and hybridization of the labeled probe to its complementary target mRNA. The tissue is incubated with the digoxigenin-labeled riboprobe for 5.5 hours at 63.5°C. Once the hybridization process is complete, the tissue is treated with a sequence of increasingly stringent washes containing decreasing salt concentrations.

Detection of the bound probe is a multi-step procedure. First, a succession of blocking steps inhibits endogenous protein activity from interfering with the colorimetric enzymatic reactions. The colorimetric reaction itself is a four part process, starting with the addition of a horseradish peroxidase (HRP)-conjugated anti-digoxigenin antibody. A Tyramide Signal Amplification (TSA) step is utilized to maximize sensitivity. The tissue is incubated with a biotin-coupled tyramide. 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**.** This results in an amplification of bound biotin molecules available for detection by up to a hundred fold (relative to the number of bound antibody molecules). These biotin molecules are then bound to neutravidin-AP. A colorimetric reaction occurs when the alkaline phosphatase (AP) conjugated to the neutravidin enzymatically cleaves the phosphate from 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and two of the resulting indoles undergo a redox reaction with nitroblue tetrazolium (NBT) to produce a blue particulate precipitate at the sites of probe binding. Once this process is completed, the tissue is treated with a wash buffer containing EDTA followed by fixation with 4% PFA. These steps stop the colorimetric reaction.

Each ISH run contains one positive (hybridized with an antisense *Drd1a* probe) and one negative control (no probe) slide. As part of our quality control process these slides are analyzed visually. The positive control slide is assessed for signal intensity, and the negative control slide is assessed for background staining (see ISH Platform Controls, http://brain-map.org/pdf/ISH_Platform_Controls.pdf). Significant deviations from the expected expression result in failure of the entire ISH run.

The ABA project *in situ* hybridization platform uses a Tecan robot with GenePaint technology developed by Dr. Gregor Eichele's Laboratory at the Max Planck Institute and Baylor College of Medicine (http://www.genepaint.org). The Allen Institute *in situ* hybridization protocol executed on the Tecan platform is detailed in Appendix I.

Figure 12 - *Customized Tecan robots are used to pipette solutions onto the slides at precisely programmed intervals, allowing the majority of the ISH procedure to be performed in an automated fashion.*

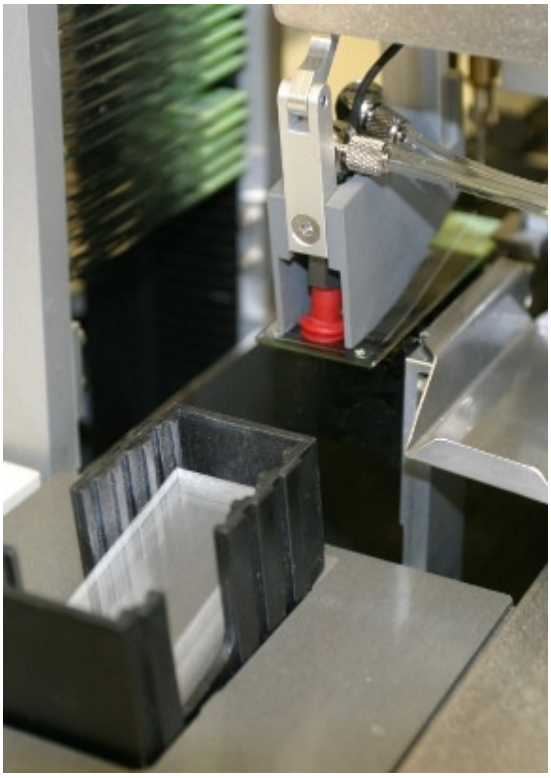

Figure 13 – *The 'gripper' of a Leica CV5030 instrument automatically applies a coverslip to a specimen slide.*

Coverslipping and Slide Cleaning Process

Immediately following the *in situ* hybridization process, slides are prepared for coverslipping. The slides are disassembled from the Tecan flow-through chambers, rinsed in water for at least 3 minutes on a Leica Autostainer XL, then coverslipped with 22 x 55 mm cover slips with Hydro-Matrix Water Solved Mounting Medium on a Leica CV5030 Coverslipper. Coverslipped slides are incubated overnight at 37°C to solidify the mounting media. Prior to scanning, the slides are cleaned to remove all excess mounting media and other debris and dust that may 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 serve as a cytoarchitectural reference to help identify specific cell populations detected on proximate series of slides processed by ISH.

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

Image Capture

The ABA project currently utilizes ten Image Capture Systems (ICS) to provide a high throughput imaging of slides. These ICS stations operate 24 hours per day, 7 days per week with minimal operator oversight other than loading slides twice per day. The scanning platform hardware and software components are outlined below. The 10 ICS units are placed on four TMC air tables to isolate the microscopes from external sources of vibration that would affect image quality. Microscope and camera function is actuated by Image Pro 5.1 with the Scope Pro/Stage Pro 5.0 Plug-In. The scanning process is controlled by the ScopeController, a custom application developed by the ABA project team.

The microscope is configured for Köhler illumination and lamp intensity is adjusted to create the brightest possible field of illumination without saturating more than 1% of image pixels on a microscope slide and coverslip. The image is white balanced and a background correction image for the 10x objective is collected using ImagePro camera controls. A background correction image is also collected for the 1.25x objective.

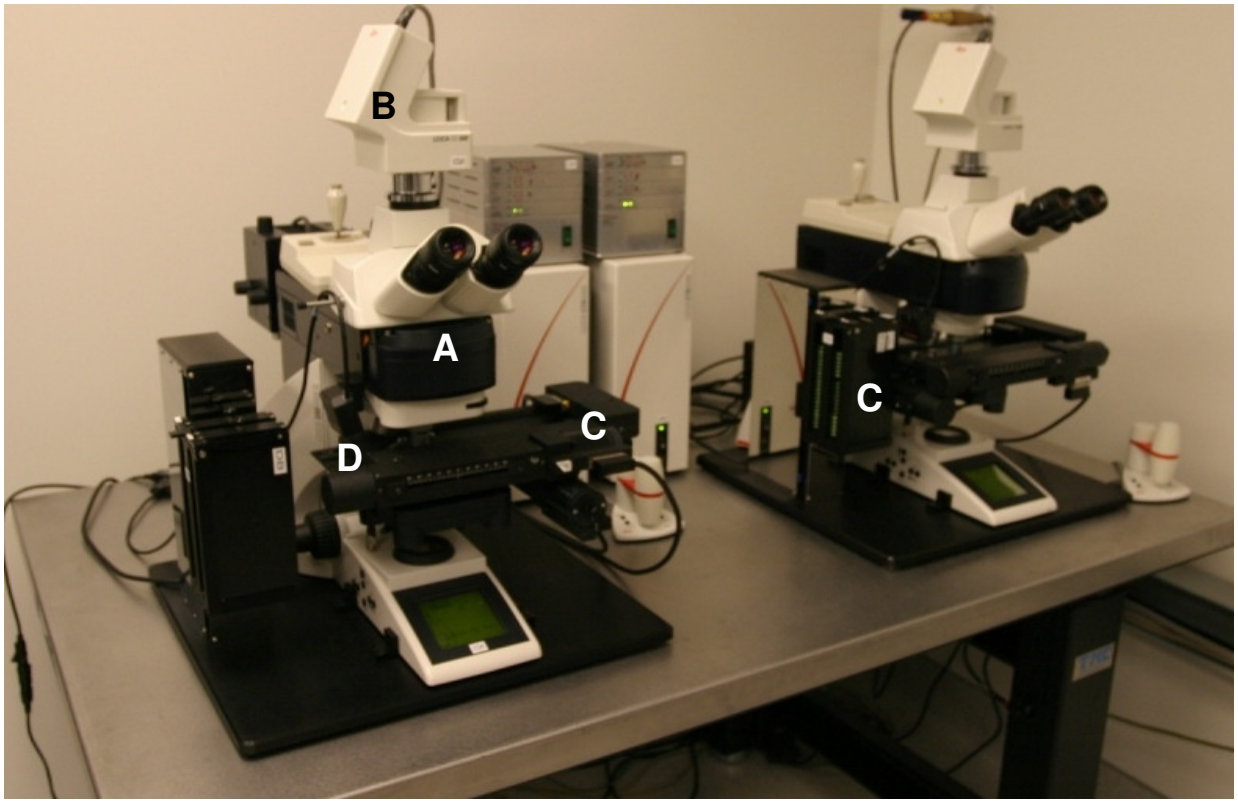

Figure 14 - *Two ICS units on a single vibration table. A) Leica DM6000B microscope and CTR MIC controller box fitted with 1.25x NA 0.04 objective and a 10x NA 0.4 Plan Apochromat objective. B) Leica DC500 camera with 0.63x tube optics. Image (tile) size is 1300 x 1030 pixels, and are captured at 24 bit depth. Image resolution is 0.95 µm/pixel (10x) and 8 µm/pixel (1.25x). C) Ludl Bioprecision x-y stage with 4mm lead screw and 50 slide loader, controlled by a Ludl MAC5000 controller. D) Microscan Barcode Scanner. Not Shown: HP xw6000-series dual processor workstation with 2GB RAM, 300 or 400GB hard drive for each microscope is housed in an IT closet. Each processor is connected to one of two KVM switches.*

Following initial calibration and set up, ScopeController performs the following operations:

1. Loads slide onto stage.
2. Scans the slide barcode and creates a local directory structure for image collection.
3. Moves off the slide and takes a series of blank images. The image series is analyzed automatically to determine if the microscope is properly color balanced and if illumination is at the proper levels to provide optimal images.
4. Captures four 1.25x tiles for each of the four brain sections on each slide. These 1.25x tiles are stitched together to make a composite image of each section. This image is then processed and a thresholding method is used to locate the tissue section on the slide.
5. Calculates a rectangular bounding box that encompasses the section.
6. Switches to the 10x objective, moves to approximately 1/3 of the way across the long axis of the tissue and performs a 2-step auto focus. In the first step, the auto focus calculates the best point of focus using a 6um step size. In the second step, the auto focus is fine adjusted using a 1µm step size.
7. Moves approximately 2/3 of the way across the long axis of the tissue and repeats the auto focus.

8. Calculates scan pattern and focus height of each tile required to completely image the section. The focus height for each tile is calculated by fitting a slope over the long axis of the tissue, with the two auto focus points serving as reference points.
9. Moves to the first tile in the scan pattern, captures background corrected images at the calculated focus height, moves to second tile, captures second image, repeats until all of the tiles are scanned.
10. Saves individual tiles locally until all of the tiles comprising that section are collected. At the conclusion of imaging the tiles, a separate copy process is spawned that uploads the image tiles and metadata files to the appropriate network location for further processing by the Informatics Data Processing (IDP) pipeline. If the network goes down for any reason, the ICS automatically continues to scan to the local drive, allowing un-interrupted image capture to continue.
11. When all four sections on each slide are scanned, the slide is replaced into the cassette and the next slide is loaded.
12. The process is repeated for each slide until all slides in the slide holder have been processed. Typical image throughput metrics are 15-20 minutes per slide.

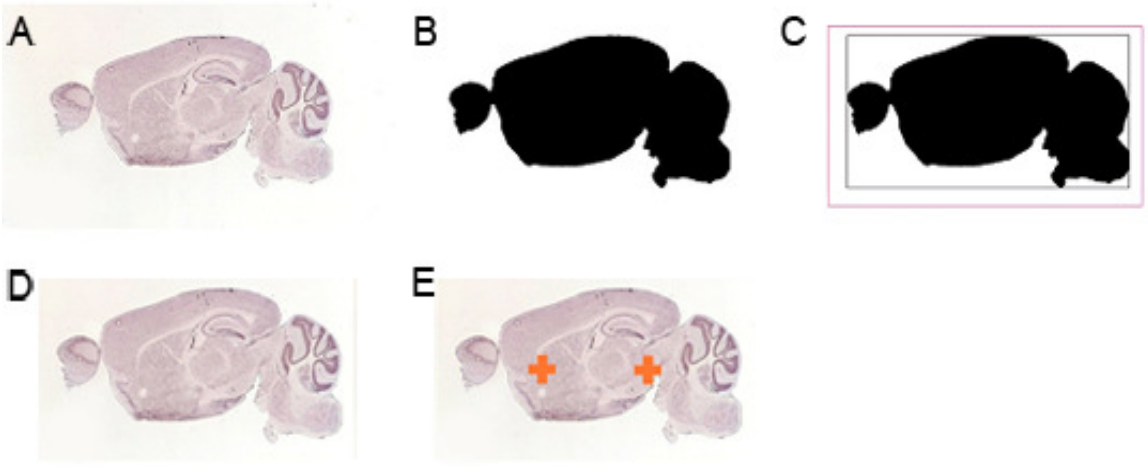

Figure 15 - *ICS tissue identification and focusing points.* **A***) Four 1.25x tiles stitched together to make a composite image of a section.* **B***) Thresholding to locate the tissue section on the slide.* **C***) Bounding box that encompasses the section.* **D***) Region on slide to be scanned.* **E***) Focus points 1/3 of the way across the long axis of the tissue*

Quality Control (QC) of ISH Slides and Captured Images

All images are visually inspected and given an artifact score (1 = slight, 2 = moderate and 3 = severe) which is recorded in the LIMS. Individuals trained to perform this QC step decide whether to accept or reject each image. In general, images are examined for focus, lighting, bubbles, tissue quality and foreign material trapped under the slide. Rescanning will occur if the quality of the image can be improved. Further informatics processing and public release of the data occurs only when 80% of the images within a series of slides generated for a specific probe pass this quality control step. Failure to reach the 80% criteria automatically initiates a second ISH run for that probe.

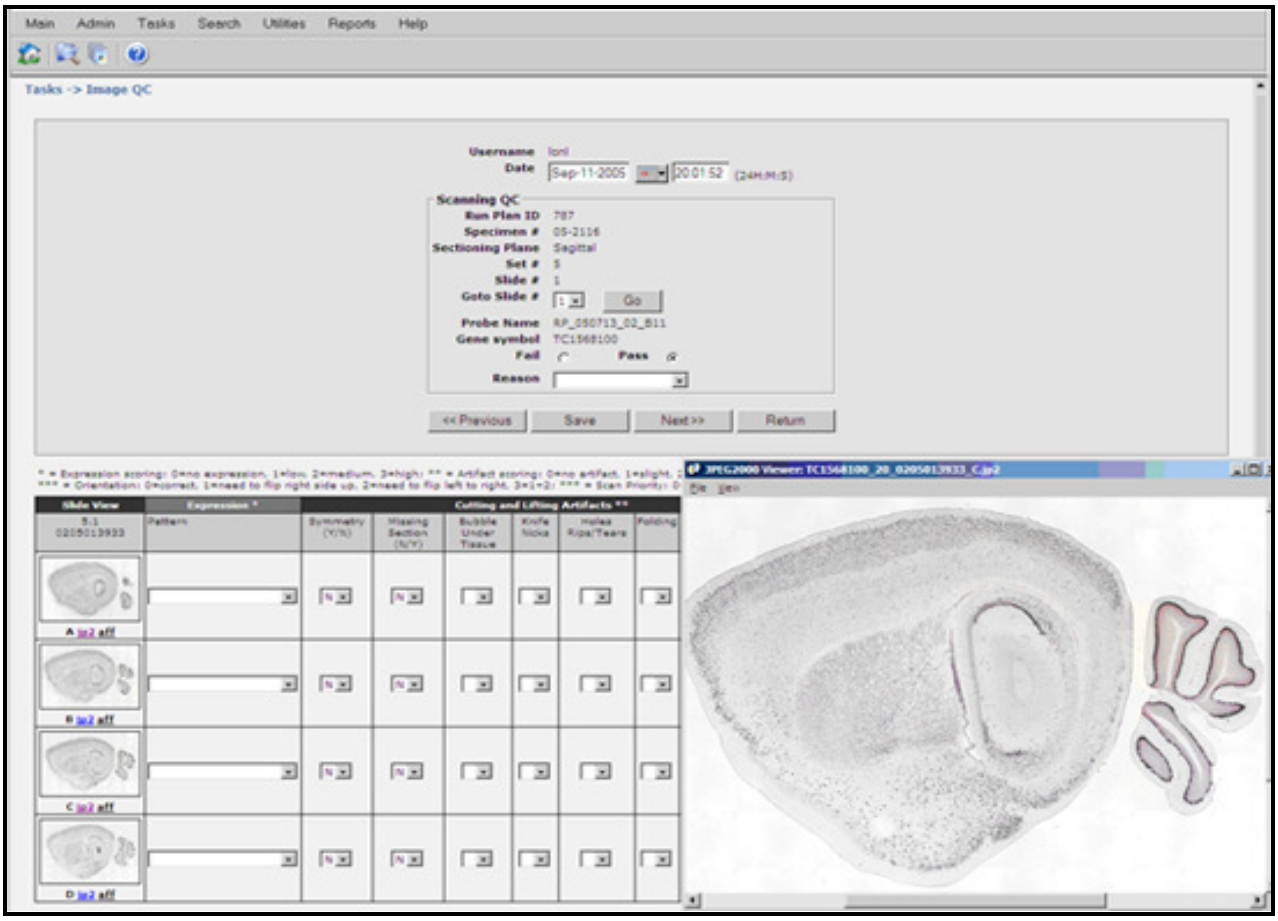

Figure 16 - *LIMS interface for viewing section images for quality control analysis.*

Appendix I – Allen Institute *in situ* hybridization Protocol

AIBS - *In Situ* Hybridization Protocol

Day 1

Cycles	Time (min)	Volume (ul)	Reagent	Temp (°C)	Time (min)	~ Run Time (hr)	Process
5	5	300	3% H2O2 in MeOH	25	25		Pre-Hyb
7	5	300	PBS (1)		35	1:00	
2	5	300	0.2M HCl		10		
4	5	300	PBS (2)		20		
1	5	400	PK(+) buffer		5	1:35	
2	10	300	Proteinase K		20		
7	5	300	PBS (3)		35	2:30	
2	10	300	4% PFA (1)		20		
7	5	300	PBS (4)		35	3:25	
2	15	300	Hyb Solution		30		
—	15	—	Ramp-up	63.5	15	4:10	
1	—	300	Riboprobe addition		—		Hybridization
1	330	—	Hybridization		330		

Day 2

Cycles	Time (min)	Volume	Reagent	Temp	Time (min)	10.00	
5	5	300	5 x SSC	61	25		Stringency Wash
5	10	350	Formamide I		50		
5	12	350	Formamide II		60	12:15	
4	8	300	0.1 x SSC (1)		24		
1	8	300	0.1 x SSC (2)	25	8		
4	5	300	NTE (1)		20		Post Hyb Blocking
3	5	300	20 mM Iodoacetamide (1)		15		
3	5	300	20 mM Iodoacetamide (2)		15	13:37	
4	5	300	NTE (2)		20		
2	5	300	TNT (1)		10		
3	5	300	4% Sheep serum (1)		15		
3	5	300	4% Sheep serum (2)		15		
4	5	200	TNT (2)		20		
2	10	300	TNB blocking buffer		20		
2	5	200	TNT (3)		10	15:27	
2	5	300	Maleate wash buffer (1)		10		
2	10	350	Blocking reagent		20		
2	5	300	Maleate wash buffer (2)		10		
2	5	250	TNT (4)		10		
3	5	350	TMN		15		
4	5	200	TNT (5)		20		
4	10	300	TNB blocking buffer		40	17:22	
2	30	350	Anti-DIG-POD		60		Colorimetric Detection
6	5	250	TNT (6)		30	18:52	
1	30	250	Tyramide-biotin		30	19:22	
6	5	300	Maleate wash buffer (3)		30		
2	20	350	Neutravidin		40		
6	5	300	Maleate wash buffer (4)		30	21:08	
4	5	250	TNT (7)		20	21:28	
2	5	400	TMN		10		
2	15	350	BCIP/NBT		30		
1	10	350	BCIP/NBT		10	22:18	
3	—	400	System liquid (1)		10		
1	—	300	NTE (3)		5		
1	10	250	4% PFA (2)		10		
1	—	400	System liquid (2)		10	23:03	

Appendix II – 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% MeOH
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)

Appendix II – Reagents used in the ISH process

Reagent	Role	Mode of Action	pH	Specs
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.

Appendix II – Reagents used in the ISH process

Reagent	Role	Mode of Action	pH	Specs
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.

Appendix II – Reagents used in the ISH process

Reagent	Role	Mode of Action	pH	Specs
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 Blking 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	Tyramine 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

Appendix II – Reagents used in the ISH process

Reagent	Role	Mode of Action	pH	Specs
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			

Appendix III- Quality Control Procedures

The ABA production process includes a number of built in Quality Control steps designed to optimize the final product and to reduce the potential for error. These steps are detailed below and in the Supplemental Document ISH Platform Controls (http://brain-map.org/pdf/ISH_Platform_Controls.pdf).

Riboprobe Generation - During probe production multiple steps are taken to ensure quality. PCR product size and homogeneity are analyzed using the Bioanalyzer. PCR products that are not of the correct size (+/- 100 bp) or that show multiple products are not used to generate probes. In addition, all PCR products generated from cDNA and genomic DNA template are sequenced from both ends, using the Forward primer and SP6.

In vitro transcription (IVT) reaction products are also analyzed using the Bioanalyzer. Those that are shorter than their predicted size are not used for ISH. 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 that are judged not to be products of secondary structure are not used for ISH.

Animals and Dissection - Each mouse is weighed before it is sacrificed. If body weight falls outside of the normal range (18.8 to 26.4g) the tissue is not used in the ABA ISH process. After dissection each brain is analyzed for gross abnormalities or dissection damage. Brains with significant abnormalities or damage are not used for the ABA ISH process.

Cryosectioning - Each slide is analyzed microscopically to ensure section quality, slides with excessive folding, tearing, or other tissue abnormalities are not used for ISH.

***In situ* Hybridization** - Each ISH run contains one positive (hybridized with an antisense *Drd1a* probe) and one negative control (no probe) slide. As part of our quality control process these slides are analyzed visually. The positive control slide is assessed for signal intensity, and the negative control slide is assessed for background staining (as described in ISH Platform Controls). Significant deviations from the expected expression result in failure of the entire ISH run.

Post *In situ* Hybridization Analysis - After image acquisition there is a quality control checkpoint involving visual inspection of scanned images for poor quality ISH, excessive artifacts, or poor imaging quality (focus, color). All images are visually inspected and given an artifact score (1 = slight, 2 = moderate and 3 = severe) which is recorded in the LIMS. In general, images are examined for focus, lighting, bubbles, tissue quality and foreign material trapped under the coverslip. Rescanning will occur if the quality of the image can be improved. Further informatics processing and public release of the data occurs only when 80% of the images within a series of slides generated for a specific probe pass this quality control step. Failure to reach the 80% criteria automatically initiates a second ISH run for that probe.

Cross Platform Validation- Our in-house scientific staff has been mining the data throughout the project and checking for concordance with other publicly available data to ensure that the expression patterns in our data match previous findings. Efforts have been made to systematically compare ABA data with other data sources, including published work and adult mouse ISH data available through another large online database, the Brain Gene Expression Map (BGEM, http://www.stjudebgem.org/). See Cross Platform Validation (http://brain-map.org/pdf/Cross_Platform_Validation.pdf) for more details on this comparison.