

ALLEN Developing Mouse Brain Atlas

TECHNICAL WHITE PAPER: OVERVIEW

ATLAS OVERVIEW

The Allen Developing Mouse Brain Atlas project serves to provide a characterization of gene expression in the brain beginning with mid-gestation through to juvenile/young adult. Building upon the foundation established by the Allen Mouse Brain Atlas, the Allen Developing Mouse Brain Atlas provides a framework to explore temporal and spatial regulation of gene expression, effectively a 4-D atlas, with a highly accessible and easily navigable free online database. The *in situ* hybridization (ISH) data encompasses approximately two thousand genes with each gene characterized across all seven developmental stages.

This project was designed in order to meet the following objectives: 1) Profile of genes functionally relevant to brain development or developmental disorders of the brain; 2) Visualization of the development of cell types and brain regions using robust neuroanatomical markers; 3) A platform to allow the user to access and understand the dynamics of gene expression through development; and 4) Easy search and navigation of the dataset to enable discovery of new genes with important roles in brain development.

PIPELINE OVERVIEW

The Allen Developing Mouse Brain Atlas utilizes the production processes as developed for the Allen Mouse Brain Atlas, a genome-scale atlas of gene expression in the mouse brain (Lein, Hawrylycz et al. 2007), with adaptations including: 1) addition of a yellow counterstain to enhance analysis of the ISH; 2) changes in tissue embedding processes for embryonic tissue; 3) adjusted proteinase K concentrations optimized for each age; 4) adjusted Nissl protocols for some timepoints; and 5) utilization of dual image acquisition platforms.

Gene Selection

There was a strong emphasis on selecting genes with known roles in brain development and genes which act as robust neuroanatomical markers to enable visualization of brain regions across development.

This gene list can be broken down into the following categories:

1. *Neuroanatomical marker genes.* Characterizing region- or cell-type specific marker genes over development can provide information about the origins of a brain region or cell type, and may help to identify precursor regions at earlier timepoints.
2. *Gene ontologies/signaling pathways relevant to brain development.* These ontologies include: axon guidance, receptor tyrosine kinases and their ligands, and genes in the Wnt Signaling and Notch Signaling pathways.
3. *Genes of general interest.* This category includes highly studied genes such as common drug targets, transcription factors, ion channels, cell adhesion, genes involved in neurotransmission, G-protein-coupled receptors, or involved in neurodevelopmental diseases, which are expressed in brain in the adult and/or in development.

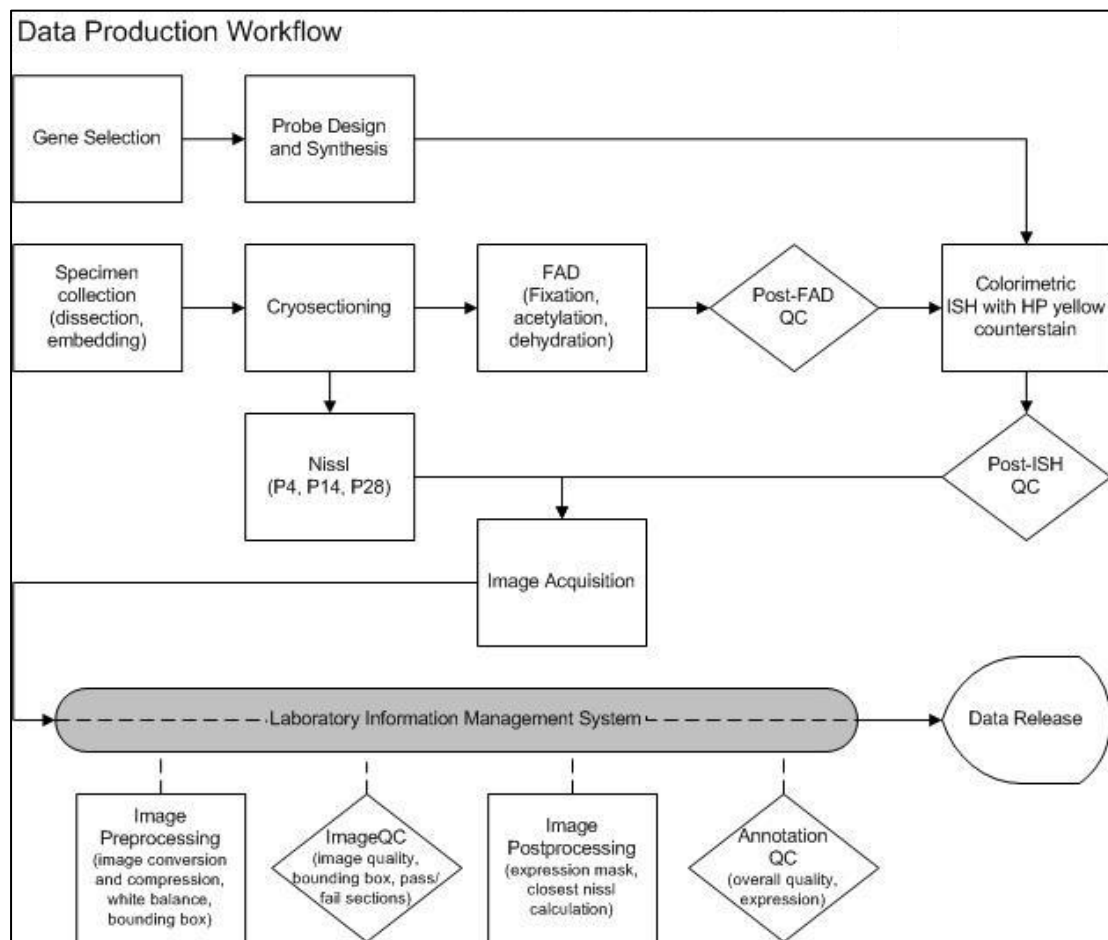


Figure 1. Workflow for data production for the Allen Developing Mouse Brain.

Probe Design and Synthesis

The procedures described in the Allen Mouse Brain Atlas [Data Production Processes](#) were used for generating probes for the developing mouse brain atlas. The two sources of probe templates were cDNA clones or pooled cDNA from mouse brain.

cDNA clones

When cDNA clones were available from the MGC (Mammalian Gene Collection, NIH), they were used as direct templates for PCR. The clones were stored as glycerol stock in 384-well and 96-well plates at -80 °C. Clone sequences were verified by comparison to RefSeq sequences. Consensus sequences with >98% homology across 80% of the total length were used to develop probes.

cDNA templates

When clones were unavailable for a given gene, pooled cDNA reactions made from mouse brain total RNA was used as a template source. Probes were generated against sequences within 3000 bp from the 3' end of the cDNA.

Mouse brain cDNA preparation

Total RNA was isolated from homogenized C57BL/6J mouse whole brain tissue using Ambion's ToTALLY RNA kit. Typical yield was 120 µg total RNA per brain. Invitrogen's Superscript III RTS First-strand cDNA synthesis kit was used for cDNA reactions in a 96-well format, using 5 µg Anchored olig-dT-25.

Primer design

Gene-specific forward and reverse primers were designed in the following way. BLAST was used to identify regions of homology in other genes/family members, and repetitive and/or homologous sequences were masked. Primer3 software was used for primer design with the following criteria:

1. Optimal size was 18-20 nt for clone templates, 22-24 nt for cDNA templates
2. GC content between 42-62%
3. PCR product size between 300-1200 nt (optimal > 600)
4. Probe location within the gene (No bias for clone templates, within 3000 bp of polyA tail for cDNA templates)

The top primer pair was chosen with the lowest penalty score. A nested reverse primer was also designed for cDNA templates. The SP6 RNA polymerase binding sequence was added to the reverse/nested primer. Primers were ordered from IDT in 96-well format at 10 μ M concentration.

All gene sequences were blasted against available collections of transcript sequences including RefSeq, MGC< Celera, TIGR, Riken, and UniGene. Regions of homology greater than 70% for regions over 100 bp were identified and excluded from probe design. (For a subset of genes in families with high homology these standards were relaxed to >90% homology over 120 bp). Within the remaining sequence, primers were designed using Primer3 software (MIT). A nested approach was used for the generation of probes from mouse brain cDNA, such that three primers were generated: a forward, a reverse, and a nested primer. An initial polymerase chain reaction (PCR) was performed using forward and reverse primers. The purified product was then used as a template for a second PCR using the same forward primer with the nested primer. When a cDNA clone was used as a template, a single PCR reaction was used with a single set of forward and reverse primers. All PCR products generated from cDNA templates were sequenced from both ends, using the forward primer and SP6.

In vitro Transcription (IVT)

Standard IVT reactions were performed using Roche's 10X DIG RNA Labeling Mix. All reactions were done in 96-well format for 2 hours at 37°C, with 30 μ l total volume. Purified PCR products served as the template, using SP6 RNA polymerase (NEB). IVT reactions were purified using Millipore's Montage 96 filter plate, and were eluted with 90 μ l of THE (0.1 mM Sodium citrate pH 6.4, Ambion) following a 30 minute room temperature incubation. IVT reactions were quantified using RiboGreen HIGH assay (Molecular Probes) and the SpectraMax-M2 plate reader (1.0 μ l in 200 μ l total volume). 1.0 μ l of each IVT reaction was analyzed on Agilent's Bioanalyzer 2100 for size confirmation and quantification. IVT reactions were stored at -80°C.

Quality Control

PCR products were evaluated for expected size and homogeneity; PCR products with multiple products were discarded. IVT products that were shorter than their predicted size were also discarded; however, it was common to see IVT products slightly larger than their predicted molecular weights, or to see multiple peaks, due to RNA secondary structure. IVT products with multiple bands were not used for ISH unless the additional bands were determined to result from secondary structure.

Dilutions

IVT reactions were diluted to working stocks of 30 ng/ μ l with THE. For hybridization, probes were diluted to 300 ng/ml into hybridization buffer (Ambion) in 96 well plates, and were stored at -20°C until use. Each well provides probe for one ISH slide.

Specimen Preparation

The C57BL/6J mouse strain was chosen for the developing mouse brain atlas because the strain is widely used in the research community, and to permit direct comparison to both the adult P56 mouse brain and to the mouse spinal cord atlas. Seven timepoints were chosen for broad characterization of gene expression, including four embryonic timepoints and three postnatal timepoints. The embryonic timepoints were chosen to

span a wide range of developmental events in utero, beginning with embryonic day (E) E11.5 and ending with postnatal day (P) P28.

Breeding

Specimens were derived from breeding pairs for all timepoints except for P28. Breeding animals were either purchased from The Jackson Laboratory West or were derived from purchased animals. Personnel monitored the presence of vaginal plugs at 12 hour intervals (6 am and 6 pm). In order to harvest embryonic specimens with accuracy to 0.5 days, only dams with visible plugs at 6 am were used to obtain embryonic timepoints. For postnatal timepoints, births were recorded at 12 hour intervals (6 am and 6 pm). Animal handling was reduced as much as possible for P4 and P14, and animals were maintained on a 12 hour light-dark schedule. The P28 animals were either bred in house and weaned at day 21, or obtained from Jackson labs, with shipping at P21, receipt of animals at P23, and maintained under normal housing conditions for 5 days prior to dissection.

Specimen Criteria

In order to obtain the most homogenous set of specimens for each timepoint, certain criteria were established (see Table 1). For each embryonic timepoint, specific Theiler stages (TS; (Theiler 1989) were chosen for production, representing the most prevalent Theiler stage occurring at that age, as determined using major criteria from Karl Theiler. For E11.5 and E13.5 embryos, gender was not determined. Male specimens were used for E15.5 through adult. For P14 collection, all pups within the harvested litter displayed eye opening.

Table 1. Specimen criteria.

Age (d)	TS	Major criteria	Gender (method)	Specimen type
E11.5	TS19	Clear anterior footplate; No sign of fingers; Pigment not uniformly distributed around eye	Undetermined	embryo
E13.5	TS21	Anterior footplate indented; Posterior footplate beginning to indent; fingers not yet separated distally; Pinna forms crest pointing 90 degrees out of head	Undetermined	embryo
E15.5	TS24	Eyelids closing; Anterior part of the back is straight; Nail primordial on toes; Head is rounded not angular	Male (tail-tip, genotype)	embryo
E18.5	TS26	Long whiskers; Pinna fused to skin; Males are showing a lump and dark pigment between the anus and genitals	Male (visual)	brain
P4	N/A	Body weight > 1.8 grams	Male (visual)	brain
P14	N/A	Body weight > 6 grams; Eyes open	Male (visual)	brain
P28	N/A	Body weight > 10 grams	Male (visual)	brain

Specimen embedding

Whole embryos (E11.5, E13.5, and E15.5) were dissected into individual wells of a 12-well plate containing chilled 4% sucrose/PBS for 15 min. During the sucrose equilibration, embryos were analyzed under a dissecting scope to determine Theiler stages for individual embryos. Embryos meeting the Theiler stage criteria were then stepped through 50:50 OCT: 4% Sucrose/PBS for 2 min prior to freezing in OCT in a dry ice

alcohol bath. E18.5 and postnatal specimens were examined for basic specimen criteria, and the brain was dissected and frozen directly in OCT. Brains were frozen at -80 °C prior to sectioning.

Cryosectioning

Specimens were sectioned at 20-25 µm (See Table 2) on a Leica 3050 S cryostat with adjacent sections placed across different slides to produce series of slides such that when a given series was used for ISH or Nissl it would result in the sampling shown in Table 2. Nissl series were generated for every specimen at ages P4, P14, and P28, and these associated Nissl images are available on the website.

Table 2. Sectioning schemes.

Ages	Section width	Sampling per gene	Nissl sampling
E11.5	20 µm	80 µm	N/A
E13.5	20 µm	100 µm	N/A
E15.5	20 µm	120 µm	N/A
E18.5	20 µm	140 µm	N/A
P4	20 µm	160 µm	160 µm
P14	25 µm	200 µm	200 µm
P28	25 µm	200 µm	200 µm

Fixation, Dehydration, Acetylation

Fixation, dehydration, and acetylation (F/A/D) were performed as described for the Allen Mouse Brain Atlas ([Data Production Processes](#)). For E11.5, E13.5, E15.5, P14, and P28 tissue, sections were allowed to air dry on slides for 30 minutes prior to F/A/D. For E18.5 and P4 tissue, the tissue was first air-dried and then baked overnight in a 37 °C oven prior to F/A/D, in order to improve tissue adhesion to the slide. The tissue was then fixed in 4% neutral buffered paraformaldehyde (PFA) for 20 minutes and rinsed for 3 minutes in 1x PBS. Acetylation was performed to reduce non-specific probe binding to tissue sections. The tissue was equilibrated briefly in 0.1 M triethanolamine and acetylated for 10 minutes 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. Finally, each slide was analyzed microscopically to ensure section quality. Slides that passed QC were stored at room temperature in Parafilm-sealed slide boxes for up to one month.

In situ Hybridization

In situ hybridization (ISH) processes were performed as described in the Allen Mouse Brain Atlas ([Data Production Processes](#)), using a non-radioactive, digoxigenin (DIG) based technique to label cells expressing a particular mRNA sequence.

The ISH protocol was performed using a Tecan robot with GenePaint technology developed by Dr. Gregor Eichele's Laboratory at the Max Planck Institute and Baylor College of Medicine. The ISH protocol executed on the Tecan platform is detailed in Appendix I. Slides were integrated into flow-through chambers on a temperature-controlled rack, and placed on a Tecan Genesis liquid handling platform. Reagents were applied using a liquid handling system. Prior to hybridization, the fixed, acetylated, and dehydrated tissues underwent steps designed to block endogenous peroxidase activity and to increase permeability of the tissue, allowing penetration and hybridization of the labeled probe to its complementary target mRNA. The tissue was incubated with digoxigenin-labeled riboprobe for 5.5 hours at 63.5 °C. Once hybridization 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 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 to HRP into a highly reactive oxidized intermediate which binds rapidly and covalently to cell-associated proteins at or near the HRP-linked probe, resulting in amplification of bound biotin molecules available for detection by up to a hundred fold. These biotin molecules are then bound to neutravidin-AP. A colorimetric reaction occurs when the alkaline phosphatase (AP) conjugated to 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-purple precipitate at the site of probe binding. Once this process was completed, the tissue was treated with a wash buffer containing EDTA followed by fixation with 4% PFA to halt the colorimetric reaction.

The major modification to the original protocol was the optimization of proteinase K concentrations to obtain the highest in situ hybridization signal while retaining tissue integrity. The proteinase K concentrations are listed in below (Table 3); postnatal timepoints P14 and P28 were processed under the same conditions as the P56 tissue in the Allen Mouse Brain Atlas.

Table 3. Proteinase K treatment.

Ages	Proteinase K (U/mL)	Time in Proteinase K
E11.5	0.0014 U/mL	1 x 10 min
E13.5, E15.5, E18.5, P4	0.0025 U/mL	1 x 10 min
P14, P28, P56	0.0175 U/mL	2 x 10 min

Every ISH run had three control slides on age-matched tissue: 2 positive controls and 1 negative control. Positive control genes were selected by the following criteria: 1) expression at all timepoints examined; 2) expression across many brain regions, such that most brain sections will show evidence of expression; and 3) expression of varying intensity at each timepoint including areas of no expression, low/medium expression, and high expression. Positive controls are Cannabinoid receptor 1 (*Cnr1*) and Calbindin 2 (*Calb2*).

Yellow Counterstain

The Feulgen-HP yellow DNA counterstain is a nuclear stain that adds definition to the tissue for the purpose of analyzing and understanding the gene expression data. This counterstain was used in conjunction with ISH for all data produced for the Allen Developing Mouse Brain Atlas, except for P56, in order to provide tissue context to the ISH signal which is otherwise difficult to discern due to the very light tissue background for embryonic ISH. The counterstain also enabled better tissue detection and focus during automated image acquisition.

After colorimetric ISH was completed on the Tecan robots ([Data Production Processes](#)), the slides were removed and underwent an acid alcohol wash (70% ethanol adjusted to pH 2.1) to reduce background, 5N hydrochloric acid washes to prepare the tissue for HP yellow counterstain, followed by HP yellow counterstain (Catalog #869, Anatech Ltd) and two final acid alcohol washes to remove non-covalently bound HP yellow. Slides are coverslipped using Hydro-Matrix Mounting Medium. The yellow counterstain and acid alcohol washes were carried out using a Leica CV5030 coverslipper. Coverslipped slides were incubated overnight at 37 °C to solidify the mounting media. Prior to scanning, slides were cleaned to remove excess mounting media and other debris.

Nissl staining

Nissl staining is a brain-specific histological technique that labels Nissl substance, the ribosomal RNA associated with rough endoplasmic reticulum. In adult and postnatal brains, Nissl staining serves as a cytoarchitectural reference to help identify specific cell populations in the brain; however, at earlier times in brain development, this stain gives no more information than a nuclear stain, such as the Feulgen-HP yellow counterstain present on all ISH datasets. Nissl sets were generated for every P4, P14, and P28 specimen at 160, 200, and 200 microns, respectively.

There are a variety of dyes that stain Nissl substance, including thionin and cresyl violet. The nissl protocol using 0.25% thionin stain described in the Allen Mouse Brain Atlas [Data Production Processes](#) was used for P14 and P28 tissue. For P4 tissue, the only modification that was made to the protocol was the substitution of 0.72% cresyl violet/60 mM sodium acetate, pH 3.4 for the thionin stain.

Briefly, after sectioning, a set of slides from each P4, P14, and P28 brains was baked at 37°C for 1-5 days. Sections were defatted with xylene substitute Formula 83 and hydrated through a graded ethanol series (100%, 95%, 70%, and 50% ethanol). After incubation in water, slides were stained in either thionin or cresyl violet, and differentiated and dehydrated in water and a graded ethanol series (50%, 70%, 95%, and 100% ethanol). Finally, slides were incubated in Formula 83 and coverslipped in DPX mounting medium. Slides were air-dried in a fume hood at room temperature.

Image Acquisition

Slides for P14, P28, and P56 tissue were scanned on the same Image Capture System (ICS) platform developed for use for the Allen Mouse Brain Atlas ([Data Production Processes](#)). All other timepoints (E11.5, E13.5, E15.5, E18.5, P4) were scanned using the ScanScope® automated slide scanner (Aperio Technologies, Inc; Vista, CA) equipped with a 20x objective and Spectrum software, and whole slide images were downsampled to a resolution of 1.0 µm/pixel.

Data Processing

The automated image processing workflow leverages the Informatics Data Processing pipeline generated for the Allen Mouse Brain Atlas (Refer to the [Informatics Data Processing](#) paper) with specific informatics modules created for this project. Following image acquisition on the ICS platform, individual section images were “stacked” or combined into a single slide image, which then entered the same pipeline as the Aperio images. All slide images were white-balanced, and a tissue detection algorithm assigned bounding boxes to individual tissue sections, which were manually assessed and adjusted when necessary. A segmentation algorithm created the expression mask, which is provided as a colorized view of expression levels across the tissue. The position of each section in the brain or specimen was calculated to a master section index which provides a framework for section position across all timepoints. For P4, P14, and P28, the closest nissl section was calculated for each ISH section.

Quality Control

Quality control measures were implemented throughout the process. There was a quality control step for section quality post-fixation just prior to ISH. Post-ISH quality control consisted of examination of both positive and negative control slides as well as a random sampling of experimental slides. After image acquisition, image quality was assessed for focus and bounding box. Finally, the annotation team provided the last measure of quality control to ensure that the data was of sufficient overall quality for release.

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