

TECHNICAL WHITE PAPER: ALLEN SPINAL CORD ATLAS

OVERVIEW

The Allen Spinal Cord Atlas is an interactive database of gene expression mapped across all anatomic segments of the cord at postnatal days 4 and 56. The indexed set of images for the entire genome is based on RNA *in situ* hybridization data, and is searchable and sortable by gene, age, expression, and in the case of the adult, by the cervical, thoracic, lumbar, sacral, and coccygeal segments. Over 80 structures in the cords can be identified with the associated high-resolution Allen Spinal Cord Reference Atlas. This resource represents an unprecedented platform for exploring the spinal cord at the cellular and molecular levels.

KEY FEATURES OF ATLAS

Current Application

- Free, public online access
- Gene expression mapped at cellular resolution by RNA *in situ* hybridization (ISH)
- C57BL/6J male mouse chosen for baseline spinal cord atlas, as in Allen Mouse Brain Atlas
- Time points postnatal day 4 (P4) for neonate/juvenile and postnatal day 56 (P56) for adult
- Genome-wide coverage of ~17,000 genes (protein coding RNAs only)
- One slide per probe
- Coronal sections (20 μ m) of cervical, thoracic, lumbar, sacral, and coccygeal segments
- Adult and neonate/juvenile sections anatomically matched on each slide and for each probe
- Sampling interval of ~2 mm throughout adult cord and ~1 mm for neonate/juvenile column for each gene
- Tissue arrays of 4 rows of 4 sections/row for adult and neonate/juvenile, for a total of 32 sections/slide
- Associated Nissl sections cut at every 200 μ m
- Adult sections annotated at the segment level: cervical, thoracic, lumbar, sacral, coccygeal
- Neonate/juvenile sections annotated at the level of spinal column with no further division
- Gene expression in adult and neonate/juvenile sections annotated at the level of detected or not detected
- Anatomic reference drawings for adult and neonate/juvenile (Allen Spinal Cord Reference Atlas)
- Interesting genes for adult and neonate/juvenile represented in 11 expression categories

OVERVIEW OF PRODUCTION

High-Throughput Processes

The systems developed for processing tissue, RNA *in situ* hybridization (ISH), Nissl staining, image acquisition, and data processing for the generation of the Allen Mouse Brain Atlas were used with significant modification at nearly every stage of the production pipeline, including specimen acquisition, dissection, embedding, sectioning, ISH, image acquisition, data processing, annotation, and web application design. The basic framework, workflow, and equipment are described in Lein et al., 2007, as well as at Data Production Processes in the Supplementary Materials section of the Allen Mouse Brain Atlas, and the modifications are described below for each step. Quality control procedures for every stage of production are governed by standard operating procedures with revision control. A spinal cord atlas advisory team was formed, and these

experts, acknowledged in the companion document Scientific Acknowledgements, provided guidance during the development of new procedures.

The ISH platform was validated for the Allen Mouse Brain Atlas with probes detected colorimetrically by comparison with the same or similar probes detected radioactively (Lein et al., 2007; Cross Platform Validation document on the Allen Mouse Brain Atlas). The chemistry was optimized to detect low signal levels and therefore the ISH reaction product is saturated in many cases. Colorimetric ISH is semi-quantitative at best and is not an absolute measurement of gene expression levels; it is relative to mRNA levels in the range of non-saturating conditions.

Specimen Selection and Acquisition

The C57BL/6J male mouse was chosen for the spinal cord atlas, because the strain is widely used in the research community and was used in the Allen Mouse Brain Atlas. The gene expression patterns identified for the C57BL/6J spinal cord will serve as a baseline for more focused studies involving transgenic stains and disease models conducted by other investigators.

Two time points were chosen for the atlas, one at P4 for the neonate/juvenile and one at P56 for the adult. Inclusion of two developmental time points and all segments of the cord will support some studies of the developing mouse, and facilitates a direct comparison between the time points and segments that manifest ALS and that which do not. Inclusion of additional developmental ages is beyond the scope of this atlas.

Adult mice were purchased from The Jackson Laboratory West, and maintained in the animal care facility for at least four days prior to cord isolation. To generate sufficient numbers of neonates/juveniles, about 50 active breeding pairs were set up so that 20-30 spinal columns could be isolated each week of operations. Personnel checked for births at 6 am and 6 pm, seven days a week, and isolated columns from neonates/juveniles at 9-10 am on post-natal day 4, so the youngest animals used are estimated to be ~P3.7 (born ~5 pm on post-natal day 0) and the oldest are estimated to be ~P4.6 (born ~7 pm on post-natal day -1), although most animals were born at night limiting the number of animals that are less than P4. Statistics such as body weight and age were collected for each animal.

Dissection, Embedding, and Sectioning

Fresh adult cords were isolated by laminectomy of C1-T5 followed by extrusion. Fresh columns were isolated using standard dissection procedures.

To maximize the efficiency of sectioning and use of the surface area on 1" x 3" glass slides, the adult cord was cut into four pieces, embedded into a single block, and four cryosections were collected from each block onto each slide of one set of slides to create tissue arrays (Figure 1). A similar procedure was used for the processing of neonate/juvenile columns. The spinal cord advisory team recommended that the entire cord and column be represented in the atlas, rather than short pieces isolated from the enlargement areas and significant other levels. Therefore, the full-length cord or column from cervical level 1 (C1) to the coccygeal levels was cut into four pieces of equal length, with the intention that particular regions such as the spinal nucleus of the bulbocavernosus, which is the anatomical homologue of Onuf's nucleus in human, would not fall at the cut sites.

Methods and tools were developed to minimize the tissue loss at cut sites, including the fabrication of cutting tools, one for neonate/juvenile columns and one for adult cords, each equipped with multiple ultra-thin feather blades. Soft agar cutting surfaces cast with a die in the shape of the adult cord were coated with saline to support the fresh, unfixed tissue during cutting. Isolation of fresh tissue for ISH, as done for brains in the Allen Mouse Brain Atlas, provided the highest level of ISH signal relative to background levels, so the mice were not perfusion fixed prior to dissection. Neonate/juvenile columns were much easier to isolate than fresh neonate/juvenile cords, and columns were rapidly frozen on dry ice to eliminate the retraction of the cord within the column inherent in the cutting of fresh columns. The neonate/juvenile columns were frozen on aluminum foil with the cervical inflexion flattened. Freezing the columns facilitated blunt cuts, and chipping or cracking of the tissue was eliminated by pre-warming the column on agar frozen at -16°C.

The pieces of fresh cord or frozen column were laid onto a ~1-2 mm layer of partially-frozen OCT, aligned using grids and a dissection microscope, and overlaid with ~1-2 mm chilled OCT. The cryomold was frozen on a dry ice alcohol bath, labeled with specimen information, and stored at -80°C until equilibrated for sectioning at 20 µm.

One adult and two neonate/juvenile OCT blocks were sectioned onto 98 slides simultaneously, one neonate/juvenile onto even slides and one neonate/juvenile onto odd slides. If the adult cord of ~32 mm is cut into four 8 mm pieces and sectioned at 20 µm, then 400 cryosections are collected, each with 4 cross sections (approximately cervical, thoracic, lumbar, sacral/coccygeal) for a total of 1600 cross sections, which fit on ~100 slides. There are 4 adult cryosections per slide, separated by (100 slides x 20 µm) 2 mm. If each neonate/juvenile column of ~16 mm is cut into four 4 mm pieces and sectioned at 20 µm, then 200 cryosections are collected, each with 4 cross sections approximately cervical, thoracic, lumbar, sacral/coccygeal) for a total of 800 cross sections, which fit on ~50 slides. There are 4 neonate/juvenile cryosections per slide, separated by (50 slides x 20 µm) 1 mm. See Figure 1 for details.

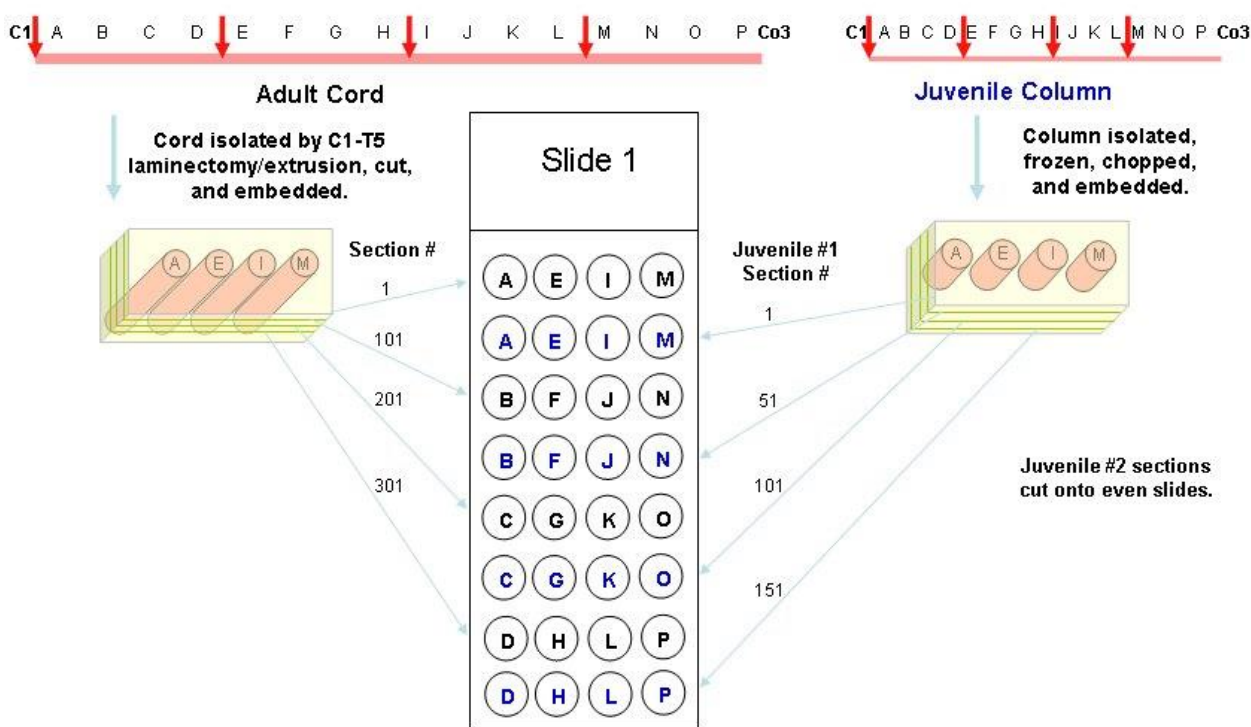


Figure 1. Dissection, embedding, and sectioning of adult cords and neonate/juvenile columns to create tissue arrays on slides.

Fixation, Dehydration, Acetylation

The procedures described in the Allen Mouse Brain Atlas Data Production Processes were used without modification.

Nissl Staining

The procedures described in the Allen Mouse Brain Atlas Data Production Processes were used for staining 10% of the slides for each slide set of 98 slides. The 5% of the odd slides (numbers 1, 21, 41, 61, and 81) corresponded to half the adult and one neonate/juvenile, whereas 5% of the even slides (numbers 10, 30, 50, 70, and 90) corresponded to half the adult and the second neonate/juvenile. The neonate/juvenile tissue sections tended to stain darker than the adult sections on the same slide; a compromise required by the tissue arrays.

Probe Design and Synthesis

The procedures described in the Allen Mouse Brain Atlas Data Production Processes were used for generating probes for the spinal cord atlas. The number of probes was limited to the approximately 18,000 clones corresponding to protein coding RNAs that were not associated with artifactual staining (~1000 probes) in the Allen Mouse Brain Atlas. An additional ~900 probes processed in the Allen Mouse Brain Atlas were not used in the spinal cord atlas because they were identified as duplicates. The ordering of probes used in generating the spinal cord atlas was alphabetical by gene symbol.

Colorimetric *In Situ* Hybridization

The procedures described in the Allen Mouse Brain Atlas Data Production Processes were used for the ISH procedures, with the exception of the proteinase K concentration used. Proteinase K was optimized at one cycle of 10 minutes at 0.0096 U/ml, which represents a compromise between the easily digested neonate/juvenile tissue and the more robust adult tissue.

Data Processing

While the data production pipeline was significantly modified for this project, data management and image processing was based on the Allen Mouse Brain Atlas pipeline, previously described in Informatics Data Processing. The Laboratory Information Management System (LIMS) schema was redesigned to accommodate for spinal cord or column sectioning, tissue arrays, and slide arrangement. Unlike the Allen Mouse Brain Atlas pipeline, the LIMS was expanded to include automated data processing. The workflow is detailed in Figure 2.

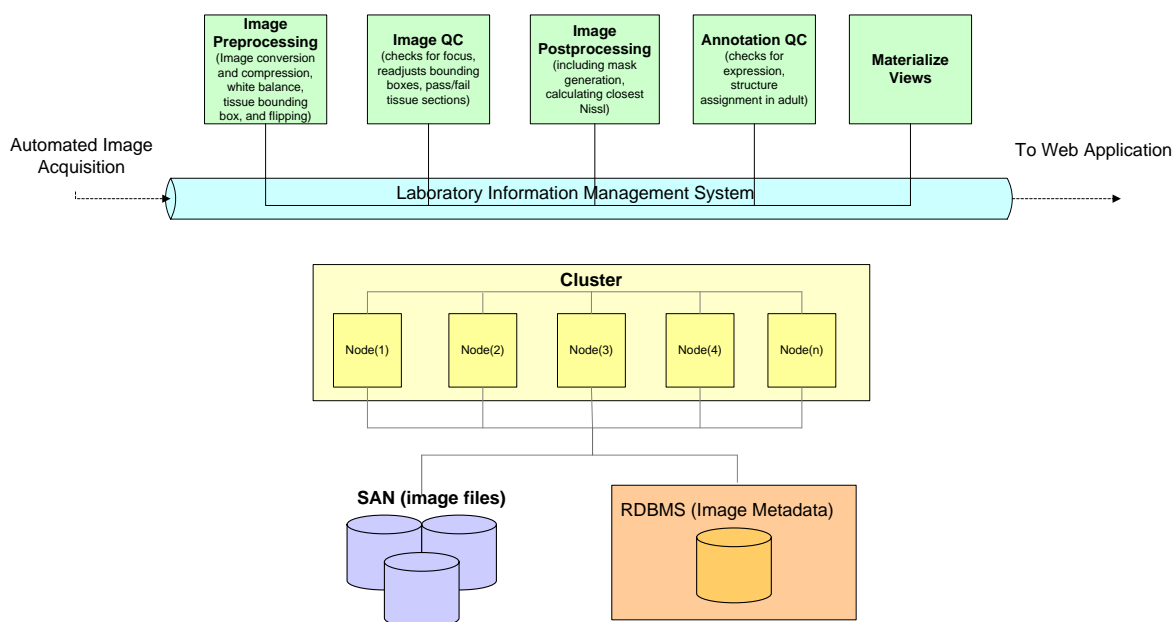


Figure 2. Allen Spinal Cord Atlas Pipeline. Image Preprocessing and Image Post-processing modules are fully automated; manual steps include Image QC and Annotation QC.

Image Acquisition

All slides were scanned with ScanScope® scanners (Aperio Technologies, Inc; Vista, CA) equipped with a 20x objective and Spectrum software, and whole-slide images were down-sampled to a resolution of 1.0 µm/pixel. During Image QC, the automated bounding box overlay was manually adjusted if necessary so that each of 32 bounding boxes per slide was placed over the correct tissue section, and images of slides with focus or tessellation problems were identified for rescanning. Subimages with failed tissue sections were failed.

Annotation

For every adult spinal cord, each Nissl-stained section was analyzed and assigned a spinal cord segment: cervical, thoracic, lumbar, sacral, or coccygeal. Both adult and neonate/juvenile Nissl image series were reviewed for section quality and poor quality sections were failed. The spinal cord segments assigned to the Nissl-stained sections were applied automatically to the ISH slides that corresponded with each closest Nissl. Neonate/juvenile columns were not annotated at the segment level. For both adults and neonates/juveniles, each ISH image was assessed for expression (yes or no), tissue quality, and ISH quality. Positive expression was determined as punctate precipitate in cell bodies above background levels. Poor quality sections and poor quality image series were failed. Image series were passed if at least one section from each region not including coccygeal was of acceptable tissue and ISH quality.

Control Sets of Genes

Tissue controls were established to monitor specimen to specimen variability and ISH run to run variability. The controls were Mest on slide 15, Rad21 on slide 36, Myoc on slide 55, Spnb2 on slide 76, and Entpd3 on slide 95. The control data and analysis will be displayed as a separate data set at the completion of the atlas.

USE OF WEB APPLICATION

The Help tab provides guidelines for:

- searching or sorting by gene, age, expression, and in the case of the adult, by the cervical, thoracic, lumbar, sacral, and coccygeal segments
- use of single image viewer and associated zooming function
- viewing nearest Nissl stained tissue section
- viewing expression mask, a binary representation of ISH signal

Image and Tissue Index Numbers

The image index is the image number in the image series from 1 to 16 for adult cords and neonate/juvenile columns. The image index is proportional to the tissue index number, and is in the order from rostral to caudal, so images 1-3 for most slides or probes are likely to be of tissue sections collected from C1 to C8 of the cervical segment, and images 14-16 for most slides or probes are likely to be of tissue sections collected from sacral or coccygeal segments.

The tissue index is the tissue section number for a particular specimen. Since the length of the cord or column varies from specimen to specimen, the maximum number of tissue sections for adults sectioned onto 98 slides is 1568 and one half of 1568, or 784 for neonates/juveniles. About 660 probes were hybridized to sections on sets of 120 slides in the initial ISH runs of the atlas project, so those adult and neonate/juvenile specimens might have a slightly higher tissue index range.

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REFERENCES

Lein ES et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445:168-176.