

BRAINSPAN

ATLAS OF THE DEVELOPING HUMAN BRAIN

TECHNICAL WHITE PAPER: REFERENCE ATLASES

OVERVIEW

The BrainSpan Atlas of the Developing Human Brain is designed as a foundational resource for studying transcriptional mechanisms involved in human brain development. The resource is the outcome of an ARRA-funded grant through the National Institutes of Health to a consortium consisting of the Allen Institute for Brain Science; Yale University; the University of Southern California; Massachusetts General Hospital, Harvard-MIT Health Sciences and Technology, Athinoula A. Martinos Center for Biomedical Imaging; the University of California, Los Angeles; and the University of Texas Southwestern Medical Center with strong collaborative support from the Genes, Cognition and Psychosis Program, which is part of the Intramural Research Program of NIMH, NIH. All data are publicly accessible via the Allen Brain Atlas data portal at www.brain-map.org or directly at www.brainspan.org.

One component of the BrainSpan Atlas is a set of neuroanatomical reference atlases generated from high quality brain specimens. To provide a novel neuroanatomical framework, full-color high-resolution Web-based digital reference atlases have been created for 15 post-conception weeks (pcw) (early mid-prenatal) whole brain, 21 pcw (mid-prenatal) cerebrum, 21 pcw brainstem, and adult whole brain. Each specimen was sectioned in the coronal plane (except for the brainstem specimen, which was sectioned in the transverse plane) and uniformly spaced sections were stained with Nissl and other histological stains. A subset of Nissl sections was then expertly annotated by Song-Lin Ding, MD, to create a digital brain or cerebrum atlas or by Gulgun Sengul, MD, to create a digital brainstem atlas. These annotations were based upon an ontology developed specifically for this project that encompasses both prenatal and post-birth brain development. The reference atlases allow users to directly compare gene expression patterns to an annotated atlas.

This white paper describes the methods and processes used to generate the reference atlases. The methods for generating other data types included in the BrainSpan atlas are described in separate technical white papers available under the [Documentation](#) tab in the online atlas.

REFERENCE ATLAS CREATION

Specimens

This work used post-mortem human brain specimens that were procured from Advanced Bioscience Resources Incorporated; Laboratory of Developmental Biology, University of Washington; NICHD Brain and Tissue Bank for Developmental Disorders, University of Maryland; and Department of Neurobiology at Yale School of Medicine (**Table 1** shows the donor profiles). All work was performed according to guidelines for the research use of human brain tissue and with approval by the Human Investigation Committees and Institutional Ethics Committees of each institute from which samples were obtained. Appropriate written informed consent was obtained and all available non-identifying information was recorded for each specimen.

Table 1. Donor profiles.

Specimen ID	Age	Sex	Race	Average RIN	Hemisphere
H376.IIIA.02	15 pcw	M	Caucasian	8	Right
H376.IV.03	21 pcw	F	Asian	9	Right
H376.IV.01	21 pcw	M	Hispanic	7	Right
H376.XI.r03	34 Y	F	AA	NA	Left

Abbreviations: AA: African-American, NA: not available, pcw: post-conception weeks, RIN: RNA integrity number, Y: years.

A protocol for this project was reviewed by Western Institutional Review Board, resulting in issuance of a Determination of Exemption.

Prenatal whole brain or cerebrum tissue was harvested and sectioned into single hemisphere coronal slabs approximately 1.5 to 2 cm in thickness. Slabs were quick-frozen in isopentane chilled to -50°C. Slabs were stored at -80°C until the time of tissue qualification and sectioning. An overview of the entire prenatal reference atlas process is shown in **Figure 1**. For the brainstem reference atlas, during tissue procurement, the brainstem was separated from the rest of the brain, resulting in a brainstem tissue block approximately 2.4 cm thick that was then subdivided into 0.4 cm and 2 cm slabs.

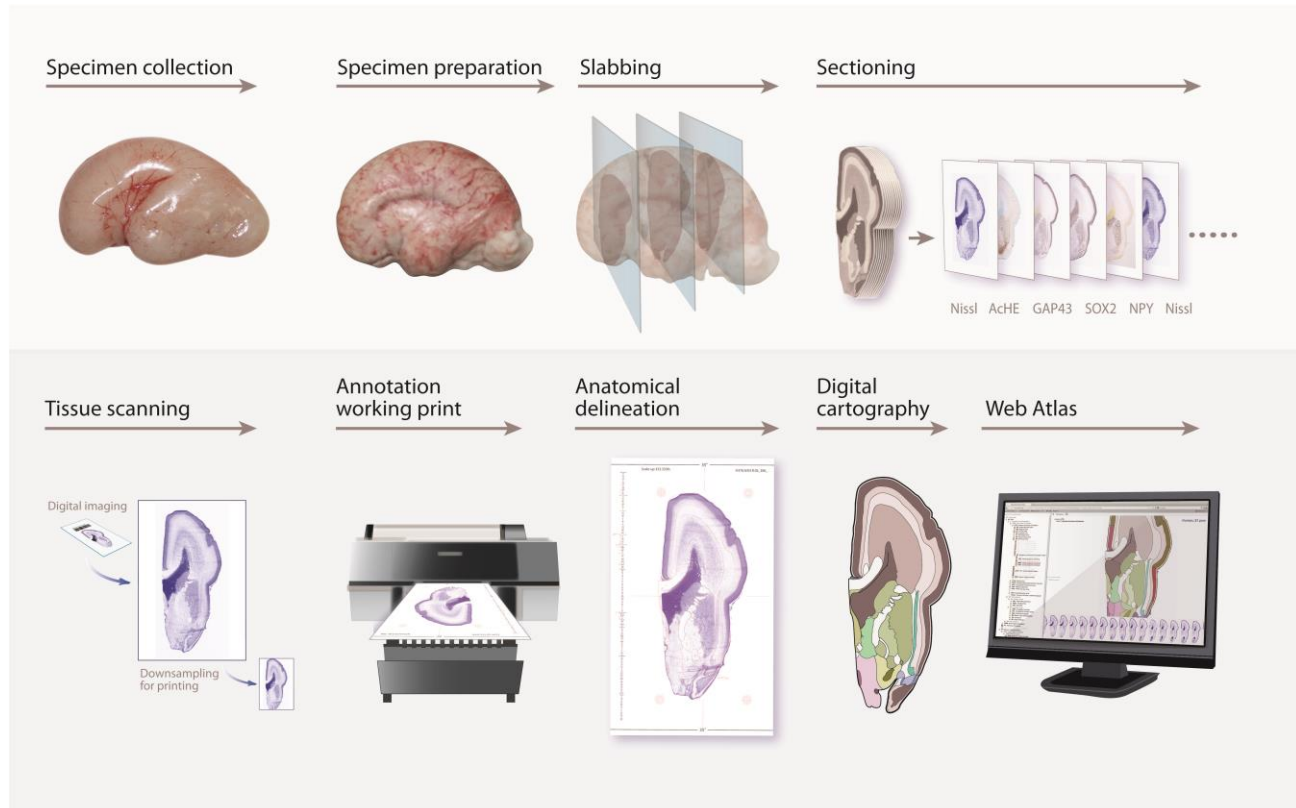


Figure 1. Overview of the prenatal reference atlas process. Fresh tissue was collected, then frozen and slabbed into 1.5 to 2 cm thick coronal slabs for whole brain and cerebrum. An entire hemisphere was sectioned in the coronal plane (20 µm thick sections). Series of slides were stained with either Nissl or acetylcholinesterase or hybridized to one of 46 selected probes (for example, *GAP43*, *SOX2* and *NPY*). All slides were scanned to produce digital images. The Nissl images were downsampled and subsequently printed. Nissl print outs were neuroanatomically delineated, digitally scanned and imported into Adobe Illustrator where polygons were created, named and colored according to the ontology. Finalized files (digital cartography) were then databased and made available on the web site.

For the adult specimen (an overview of the entire adult reference atlas process is shown in **Figure 2**), after the brain was removed from the skull, 50-100 mls of 4°C phosphate buffered saline (PBS) was injected into the carotid arteries and vertebral arteries to flush the arteries and veins. Following the PBS flush, 4%

Periodate-lysine-paraformaldehyde (PLP) was injected into the arteries, which were then ligated. Next, the brain was suspended and immersed in 4% PLP at 4°C. For incubation in PLP, a piece of string was placed through the arterial system on the ventral side of the brain and used to suspend the brain in PLP. PLP solution was replaced every three days to enhance the fixation process. Fixation progress was determined by magnetic resonance imaging. Following complete fixation, the brain was subjected to magnetic resonance imaging and diffusion weighted imaging (imaging details are located in an accompanying Technical White Paper) and then stored in PLP at 4°C until further processed.

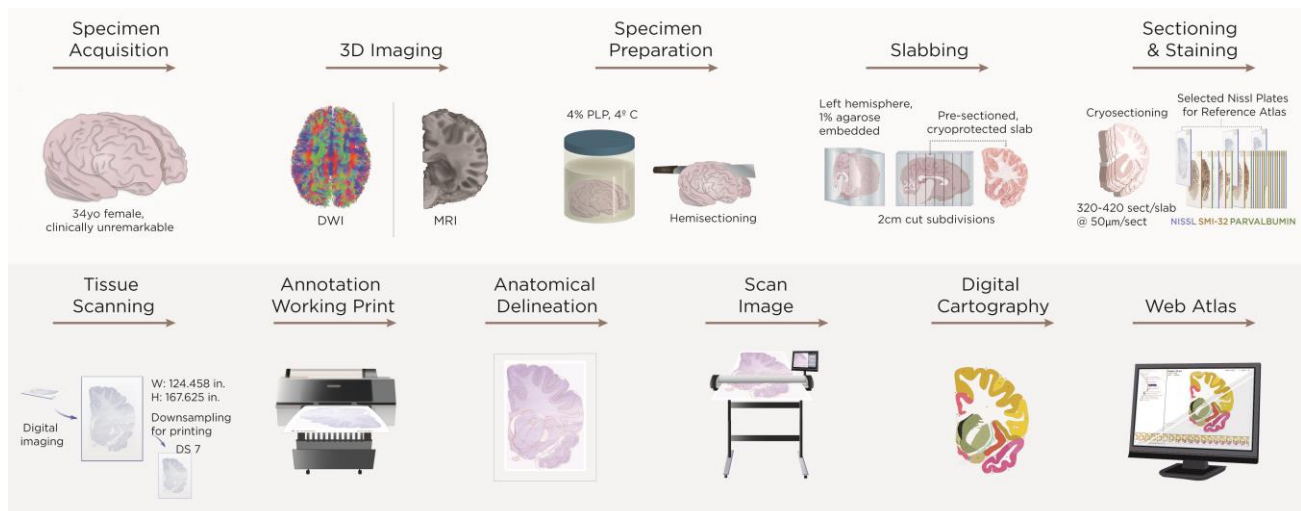


Figure 2. Overview of the adult reference atlas process. Tissue was collected, 3-D imaged, fixed, hemisected and slabbed coronally at approximately 2 cm thickness. Each slab was then serially sectioned at 20 µm thickness. Series of slides were stained with either Nissl or immunohistochemically treated to reveal SMI-32 and parvalbumin. Slides were scanned to produce high-resolution digital images. Similar to the prenatal atlas, select Nissl plates were downsampled and printed in large format for neuroanatomical delineation. Once annotated, prints were digitally scanned and imported into Adobe Illustrator where structure polygons were created, identified and colored ontologically. Finalized scalable vector graphic (svg) files were then programmatically rendered for use in the interactive web application.

The intact fixed adult brain was bisected down the midline, with special attention paid to the cerebellum and brainstem to ensure that they were equivalently divided down the center. Individual hemispheres were embedded in 1% agarose. Following agarose embedding, a flexi-slicer was utilized to cut each hemisphere from anterior to posterior into eight 2.0 cm slabs. Following photodocumentation of the individual slabs, the slabs were cryoprotected and frozen until sectioned for histological processing.

For each adult slab, the cryoprotection process started with three cycles of incubation in 4°C PBS on an orbital shaker in a 4°C refrigerator for 15 minutes (fresh PBS was used for each incubation cycle). Next, the slab was incubated in 10% sucrose-PBS on an orbital shaker in a 4°C refrigerator. The slab was removed from the solution after having sunk to the bottom of the container. Next, the slab was incubated in fresh chilled 20% sucrose-PBS on an orbital shaker in a 4°C refrigerator until the tissue sunk. Then, the slab was incubated in fresh chilled 30% sucrose-PBS on an orbital shaker in a 4°C refrigerator until the tissue sunk.

Following cryoprotection, the adult slabs were frozen in a dry ice/isopentene bath (between -50°C and -60°C). The posterior/dorsal/medial surface was marked with India ink. Each slab was placed on a Teflon coated plate, which was then submerged into the isopentene slurry ensuring that no more than 75% of the slab was submerged. After freezing was complete, the frozen slabs were photodocumented, placed in bags that were then vacuumed sealed, labeled, and stored at -80°C until further processed.

One adult slab at a time was rapidly thawed in phosphate buffer at room temperature, treated overnight with 20% glycerol and 2% dimethylsulfoxide to prevent freeze-artifacts and then embedded in a gelatin matrix using MultiBrain® Technology (NeuroScience Associates, Knoxville, TN). Co-embedded with each slab were

two or more 'matrix markers' which would serve to identify right and left as well as, by the use of different colors, to identify each slab.

After curing in a weak formaldehyde solution, the adult blocks were rapidly frozen by immersion in isopentane chilled with crushed dry ice and mounted on a freezing stage of a hydraulically driven sliding microtome (Lipshaw model 90A). The blocks were sectioned coronally at 50 μm . All sections cut (none were discarded) were collected sequentially into a 4x6 array of containers filled with Antigen Preserve solution (50% PBS pH 7.0, 50% ethylene glycol, 1% polyvinyl pyrrolidone).

Tissue Qualification

Tissue samples from prenatal donors were tested for RNA quality. For RNA quality assessment purposes, two to six tissue sample sections at 14-20 μm each were used for RNA extraction using the MELT Total Nucleic Acid Isolation System (Ambion, Foster City, CA). Samples were stored at -80C after lysis until processed in 96-well plates for RNA isolation. RNA was eluted with 20 μl nuclease-free water and stored at -80°C. Yields were typically between 1,000-3,000 ng total RNA. No further purification of RNA samples was performed.

RNA quality was determined for each prenatal specimen using high-resolution capillary electrophoresis on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) and Agilent's RIN software algorithm to generate RNA Integrity Numbers (RIN values). RNA was eluted in 20 μl nuclease-free water, standardized to a concentration of 5 ng/ μl and 1.0 μl was assayed on a Pico Bioanalyzer chip. RIN values were based on the entire electrophoretic trace and ranged from 1 to 10, where 1 corresponds to completely degraded RNA and 10 corresponds to perfectly intact RNA (Schroeder et al, 2006). In addition to RIN assessment, samples were tested for histological tissue quality by Nissl staining (described in detail below).

Nissl

For the prenatal reference atlas specimens, after sectioning 20 μm thick sections in the coronal plane from an entire hemisphere for the whole brain and cerebrum specimens and in the transverse plane for the brainstem specimen, slides were stored at 37°C for 1 to 5 days and were removed 5 to 15 minutes prior to 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 0.213% thionin, then 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 microscopically to ensure staining quality. Slides were cleaned in preparation for digital imaging.

For the adult specimen, every fourth 50 μm thick coronal section was mounted on gelatin coated 3x5 glass slides, air dried and carried through the following sequence: 95% ethanol; 95% ethanol/formaldehyde; 95% ethanol, chloroform/ether/absolute ethanol (1:8:1); 95% ethanol; 10% HCl/ethanol; 95% ethanol; 70% ethanol; deionized water; Thionine (0.05% Thionine/acetate buffer, pH 4.5); deionized water; 70% ethanol; 95% ethanol; acetic acid/ethanol; 95% ethanol; 100% ethanol; 100% ethanol; 1:1 100% ethanol/xylene; xylene; xylene; and then finally coverslipped.

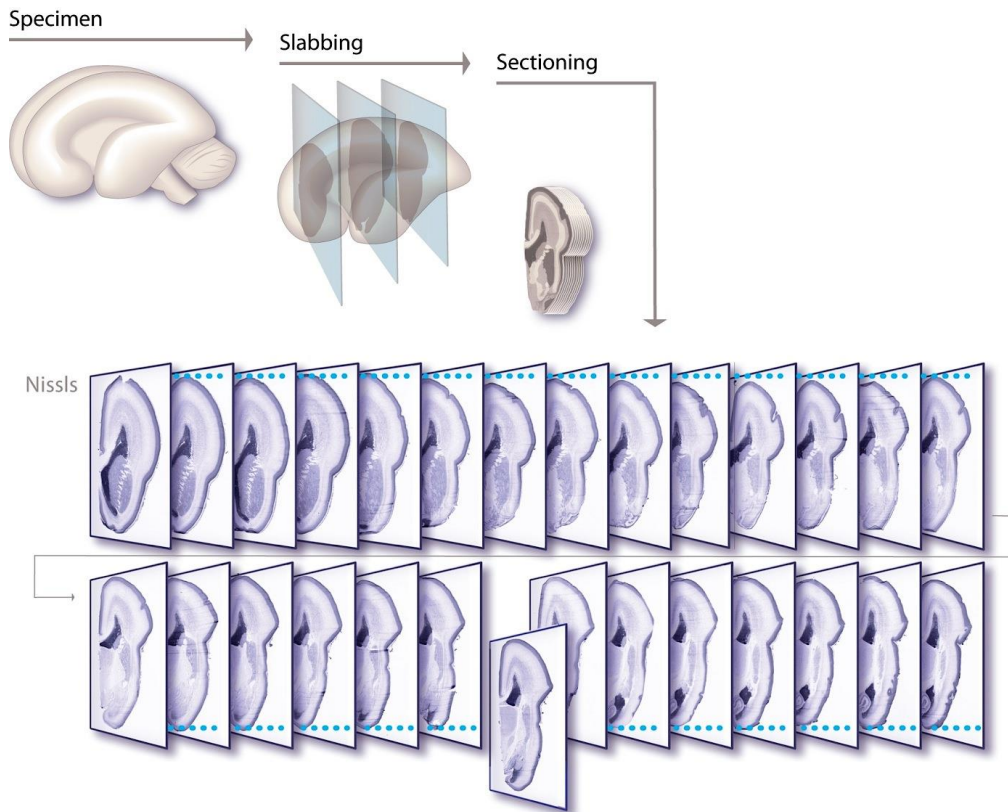


Figure 3. Prenatal whole brain and cerebrum reference atlas Nissls. After identification of the prenatal reference atlas specimens, the specimens were slabbed and cryosectioned. Nissl staining was done throughout the coronal hemisphere such that there were 3 Nissl sections approximately every 1.2 mm. A subset of Nissl sections were then selected for digital cartography.

For the H376.IIIa.02 specimen (15 pcw), two slabs were generated. A total of 115 Nissl-stained sections were produced at 1.04 mm spacing, 45 of which were chosen for annotation for this prenatal reference atlas. To reduce the sampling gap between the two slabs, a near-complete section from the trim-in of slab 2 was included, resulting in 46 total reference atlas plates: 23 from slab one (~1 mm sampling density for the first 7 Nissls, ~0.5 mm for the remaining 16 Nissls), 22 from slab two (~0.5 mm sampling density for the first 16 Nissls, ~1 mm for the remaining 6 Nissls), and a single section effectively between slabs one and two.

For the H376.IV.03 specimen (21 pcw), four slabs were generated. Each of these four slabs were sectioned into 174 Nissls with 3 Nissls per 1.2 mm. Two of these Nissl sections per 1.2 mm were located back to back so that the best atlas quality section could be chosen between the two adjacent Nissl sections (see **Figure 3**). A total of 81 Nissls were chosen for annotation for this prenatal reference atlas: 13 Nissls from slab one (~1.2 mm sampling density), 32 Nissls from slab 2 (~0.5 mm sampling density), 22 Nissls from slab 3 (~0.5 mm sampling density for the first 16 Nissls, ~1.2 mm sampling density for the remaining 6 Nissls), and 14 Nissls from slab 4 (~1.2 mm sampling density).

For the H376.IV.01 brainstem specimen (21 pcw), two tissue slabs were generated. A total of 76 Nissl-stained sections were produced at 0.24 or 0.28 mm spacing. A total of 41 Nissls were chosen for annotation for the brainstem prenatal reference atlas (approximately 0.25 mm and 0.5 mm sampling density in slab 1 and slab 2, respectively).

For the H376.XI.r03 specimen (34 years), eight slabs were generated. A total of 668 high quality Nissl-stained sections were produced at 200 micron spacing, 106 of which were chosen for annotation for the adult reference atlas: 4 from slab one (varying sampling density, 3.0 mm to 3.4 mm), 9 from slab two (varying sampling density, 2.9 mm to 3.3 mm), 5 from slab three (varying sampling density, 3.2 mm to 3.4 mm), 24 from slab four (varying sampling density, 0.6 mm to 1.2 mm), 21 from slab five (varying sampling density, 0.6

mm to 1.1 mm), 19 from slab six (varying sampling density, 0.6 mm to 1.0 mm), 19 from slab seven (varying sampling density, 0.6 mm to 1.1 mm), and 5 from slab eight (varying sampling density, 3.0 mm to 3.2 mm).

Immunohistochemistry

For immunohistochemistry for the adult reference atlas, every eighth 50 µm thick section was stained free-floating. All incubation solutions from the blocking serum onward used Tris Buffered Saline (TBS) with Triton X-100 as the vehicle; all washes were with TBS.

After hydrogen peroxide treatment and blocking serum, adult tissue sections were immunostained with the primary antibodies overnight at room temperature. Primary antibodies included SMI-32 (1:3,000 dilution) and parvalbumin (1:10,000 dilution). Vehicle solutions contained Triton X-100 for permeabilization. Following TBS washes a biotinylated secondary antibody (anti-IgG of host animal in which the primary antibody was produced, Vecta Elite horse anti-mouse, preabsorbed against rat IgG) was applied. After further TBS washes Vecta's ABC solution (avidin-biotin-HRP complex; details in instructions for Vectastain Elite ABC, Vector, Burlingame, CA) was applied. After TBS washes, sections were treated with nickel-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide to create a visible reaction product and following TBS washes, mounted on gelatin coated 3x5 glass slides, air dried, dehydrated in alcohols, cleared in xylene and coverslipped.

Acetylcholinesterase

For the prenatal reference atlas specimens, a modified acetylcholinesterase (AChE) protocol was used to help delineate subcortical structures at high resolution. Unlike AChE staining in fixed tissue, staining in fresh frozen tissue does not elucidate cholinergic fibers commonly seen in the cortex, but instead provides demarcation of various subcortical nuclei. AChE staining was performed using a direct coloring thiocholine method combined with a methyl green nuclear counterstain to improve tissue visibility (Karnovsky and Roots, 1964). Glass slides with fresh frozen tissue sections were removed from 4°C, allowed to equilibrate to room temperature, fixed in 10% neutral buffered formalin (NBF) and washed briefly in ultra-pure water. Sections were then incubated for 30 minutes in a solution of acetylthiocholine iodide, sodium citrate, cupric sulfate, and potassium ferricyanide in a 0.1M sodium acetate buffer (pH 6.0), washed in 0.1M Tris-HCl buffer (pH 7.2), incubated with 0.5% diaminobenzidine (DAB) in 0.1M Tris-HCl with 0.03% hydrogen peroxide. Slides were incubated in 0.2% methyl green, briefly dipped in 100% ethanol, cleared with Formula 83 and coverslipped with DPX.

In situ hybridization

For the *in situ* hybridization data generation for the prenatal reference atlas specimens, details of the *in situ* hybridization process are available in the *In Situ* Hybridization White Paper under the [Documentation](#) tab in the online atlas. In brief, a colorimetric, digoxigenin-based method for labeling target mRNA was used to detect gene expression on human prenatal tissue sections with 46 unique genes (listed in **Table 2**).

Table 2. Prenatal *in situ* hybridization gene list.

Gene Symbol	Gene Name	Entrez Gene ID
ARX	aristaless related homeobox	170302
CALB2	calbindin 2	794
CDH4	cadherin 4, type 1, R-cadherin (retinal)	1002
CNTNAP2	contactin associated protein-like 2	26047
DCX	doublecortin	1641
DLX1	distal-less homeobox 1	1745
DLX2	distal-less homeobox 2	1746
DLX5	distal-less homeobox 5	1749
ENC1	ectodermal-neural cortex (with BTB-like domain)	8507
EOMES	eomesodermin homolog (<i>Xenopus laevis</i>)	8320

ERBB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	2066
ETV1	ets variant 1	2115
FABP7	fatty acid binding protein 7, brain	2173
FADS2	fatty acid desaturase 2	9415
FEZF2	FEZ family zinc finger 2	55079
FOXG1	forkhead box G1	2290
FOXP1	forkhead box P1	27086
GAP43	growth associated protein 43	2596
GFAP	glial fibrillary acidic protein	2670
GRIK2	glutamate receptor, ionotropic, kainate 2	2898
HOXA4	homeobox A4	3201
LBX1	ladybird homeobox 1	10660
LHX2	LIM homeobox 2	9355
LHX5	LIM homeobox 5	64211
LMO4	LIM domain only 4	8543
MECP2	methyl CpG binding protein 2 (Rett syndrome)	4204
MET	met proto-oncogene (hepatocyte growth factor receptor)	4233
NES	nestin	10763
NKX2-1	NK2 homeobox 1	7080
NPY	neuropeptide Y	4852
NRGN	neurogranin (protein kinase C substrate, RC3)	4900
NRXN1	neurexin 1	9378
NTRK2	neurotrophic tyrosine kinase, receptor, type 2	4915
PAX6	paired box 6	5080
PLXNA2	plexin A2	5362
RELN	reelin	5649
SATB2	SATB homeobox 2	23314
SHANK3	SH3 and multiple ankyrin repeat domains 3	85358
SOX10	SRY (sex determining region Y)-box 10	6663
SOX2	SRY (sex determining region Y)-box 2	6657
SST	somatostatin	6750
SYNGAP1	synaptic Ras GTPase activating protein 1 homolog (rat)	8831
TBR1	T-box, brain, 1	10716
TPP1	tripeptidyl peptidase I	1200
VIM	vimentin	7431
ZIC1	Zic family member 1 (odd-paired homolog, Drosophila)	7545

Digital Imaging and Processing of 1x3 and 2x3 Histologically Stained Sections

Digital imaging of 1x3 and 2x3 slides was done using the ScanScope XT (Aperio Technologies Inc., Vista, CA) with slide autoloader. Final resolution of the images was 1 $\mu\text{m}/\text{pixel}$. All images were databased and preprocessed, then subjected to Quality Control (QC) to ensure optimal focus and that no process artifacts were present on the slide images. Images that passed this initial QC were further assessed to ensure that the staining data were as expected. Once all QC criteria were met, images became available for annotation of anatomic structures.

Digital Imaging and Processing of 3x5 Histologically Stained Sections

Digital imaging of 3x5 slides was done using a custom Nikon large format microscope system. The system collected hundreds of images in lengthwise strips, which are later stitched together to create one large image with 1 $\mu\text{m}/\text{pixel}$ resolution. The software NIS-Elements AR was utilized for image acquisition. The exposure time, white balance, and flat field correction were set independently for each slide. The acquisition settings for Nissl and immunohistochemistry (SMI-32 and parvalbumin) slides were 1.40 x gain, linear contrast, and 1280 x 960 resolution. Exposure times were contingent upon the microscope and were either 40 or 60 ms for Nissl, 20 or 60 ms for parvalbumin, and 20 or 60 ms for SMI-32. The files acquired by the Nikon microscopes were ND2 files, which were then subsequently converted to TIFF.

Annotation of 2-D Sections

Annotation drawings were done on the Nissl printouts by Song-Lin Ding, MD for the whole brain and cerebrum reference atlases and by Gulgun Sengul, MD for the brainstem reference atlas, and then digitally scanned. Digital cartographic translation of expert-delineated Nissl printouts was done using Adobe Creative Suite 5. The resulting vector graphics were then converted to Scalable Vector Graphics (SVG). Each polygon was then associated with a structure from the ontology: collating polygons in this way allows the flexibility to create various presentation modes (e.g., with or without colorization and transparency). The ontology was colorized to assist users with identifying structures across different sections (see **Figures 4-8**). Gross ontological groups (“parents”) were assigned hues from a range of the color spectrum, with more “primitive” structural groups receiving generally cooler hues (e.g., medulla; blue) and more “evolved” receiving warmer hues (neocortex; yellows, oranges, reds, etc). Each structure within a given parent group (“child”) was given a variation of the parent hue according to its relative cellular contrast in Nissl. The following general principal was applied: the higher the density, the deeper the shade (*i.e.*, addition of black to hue); the lower the density, the deeper the tint (*i.e.*, addition of white to hue). Large parent groups (e.g., thalamus) were assigned uniformly light variations of their principle hues to provide a visually subtle, cohesive backdrop for component substructures, which often exhibit a range of relative cellular contrasts (reflected by shades/tints).

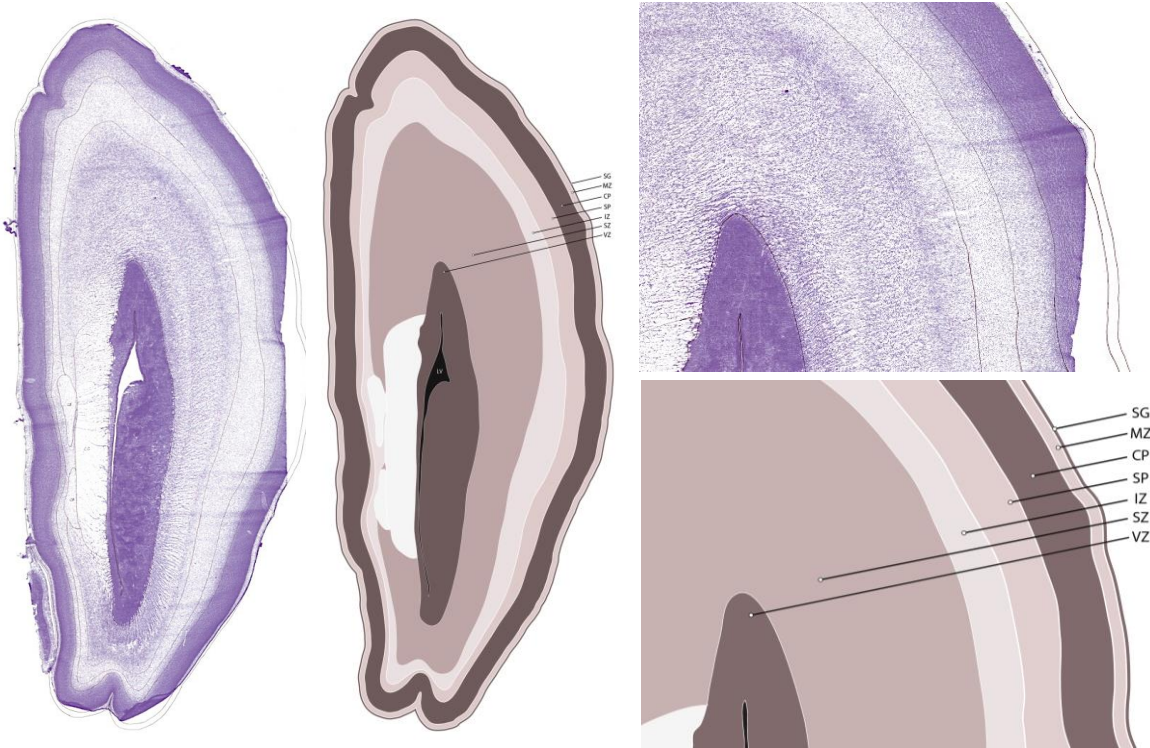


Figure 4. Prenatal cortical layer delineation. Cortical layers from superficial to deep are subpial granular zone (SG), marginal zone (MZ), cortical plate (CP), subplate (SP), intermediate zone (IZ), subventricular zone (SZ), and ventricular zone (VZ).

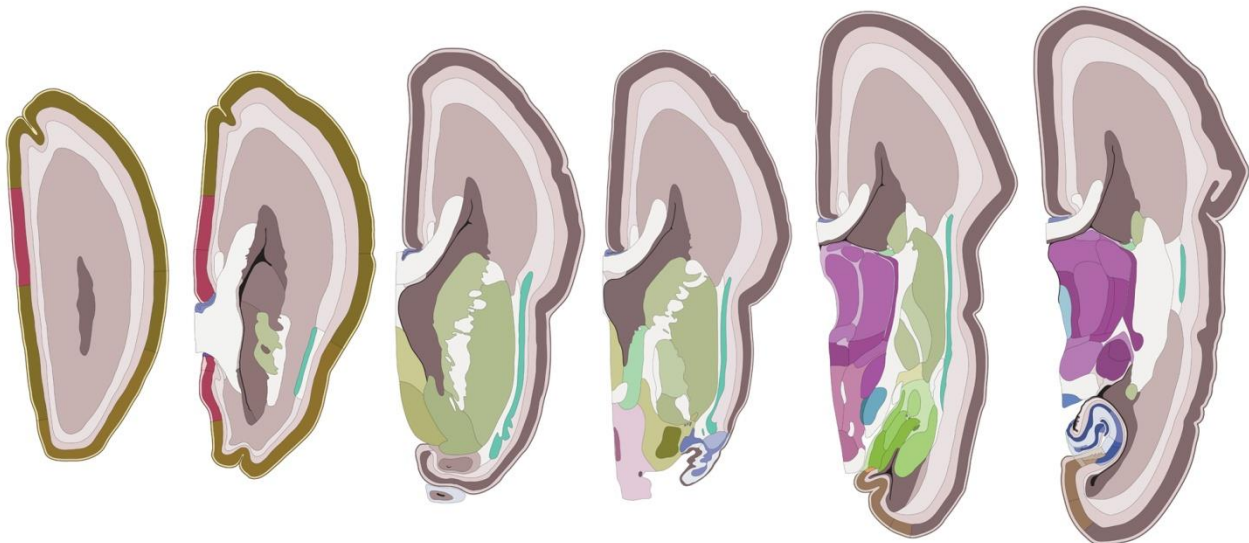


Figure 5. Reference atlas plates from the 21 pcw cerebrum reference atlas. Coronal reference atlas plates from the 21 pcw cerebrum prenatal reference atlas specimen are shown (right hemisphere). The left two plates are from slab one, the right four are from slab two. Structurally, green represents cerebral nuclei, including amygdala, basal ganglia, and basal nuclei; blue represents hindbrain, including pons, medulla and cerebellum. Purple represents thalamus, mauve represents hypothalamus, sky-blue represents midbrain, teal represents claustrum, lavender represents allocortex, tan represents periallocortex, and putty represents prenatal-specific transient layers/regions. Light gray and black represent white matter and ventricles, respectively.

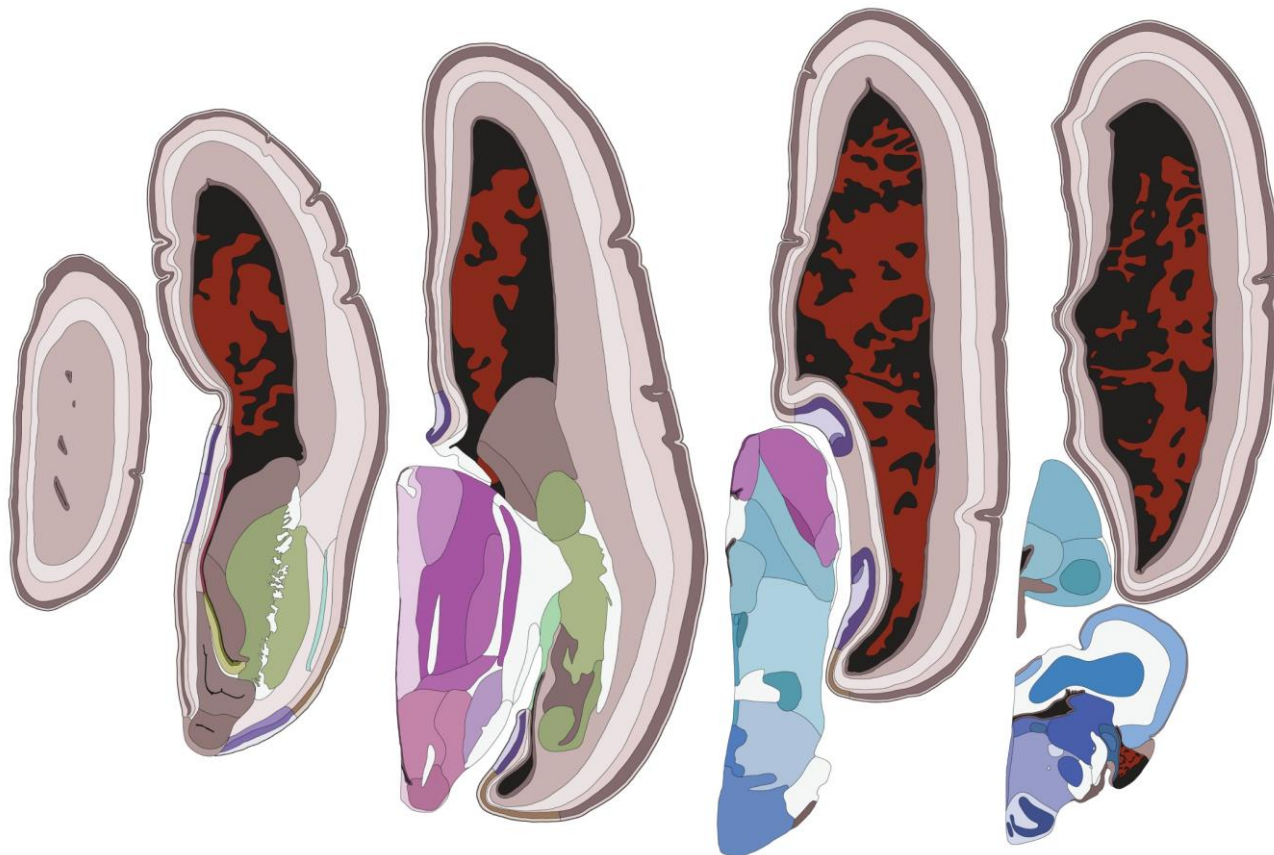


Figure 6. Reference atlas plates from the 15 pcw reference atlas. Coronal reference atlas images from the 15 pcw prenatal reference atlas specimen are shown (right hemisphere). The left two plates are from slab one, the right three are from slab two. Structurally, green represents cerebral nuclei, including amygdala, basal ganglia, and basal nuclei; blue represents hindbrain, including pons, medulla and cerebellum. Purple represents thalamus, mauve represents hypothalamus, sky-blue represents midbrain, teal represents claustrum, lavender represents allocortex, tan represents periallocortex, and putty represents prenatal-specific transient layers/regions. Light gray, black and maroon represent white matter, ventricles and choroid plexus, respectively.

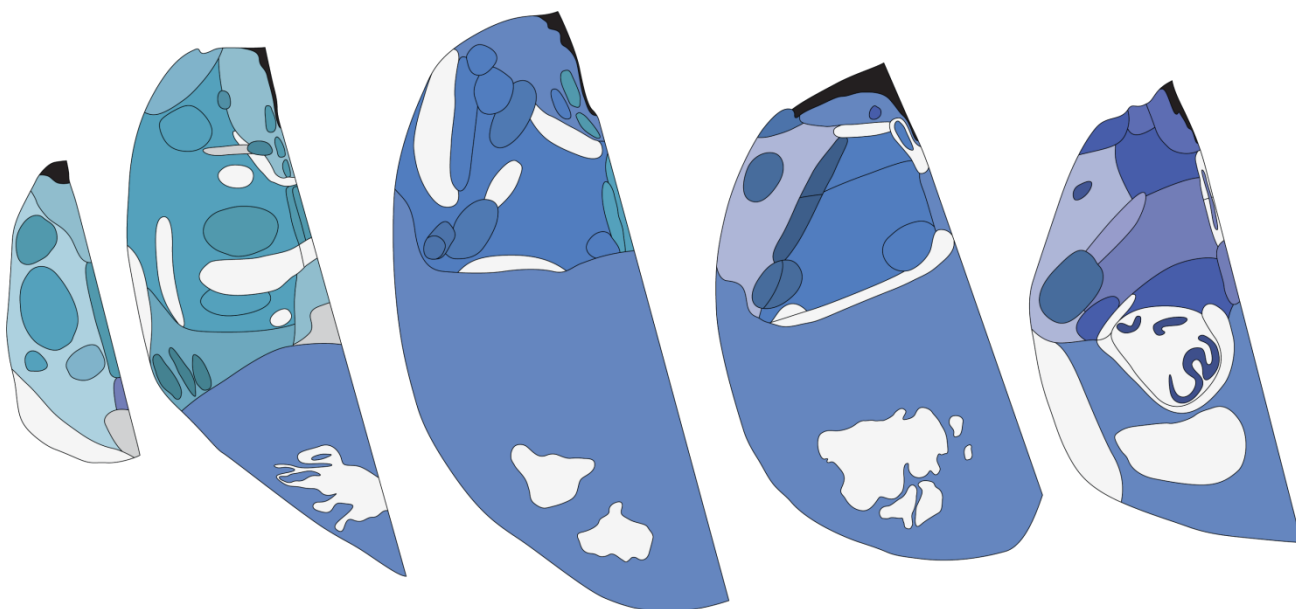


Figure 7. Reference atlas plates from the 21 pcw brainstem reference atlas. Transverse reference atlas plates from the 15 pcw prenatal reference atlas specimen are shown (right hemisphere). The left two plates are from slab one; the right three are from slab two. Structurally, sky-blue represents midbrain, cornflower-blue represents pons and royal-blue represents medulla. Light gray, medium gray and black represent white matter, ventricles, and nerve roots, respectively.

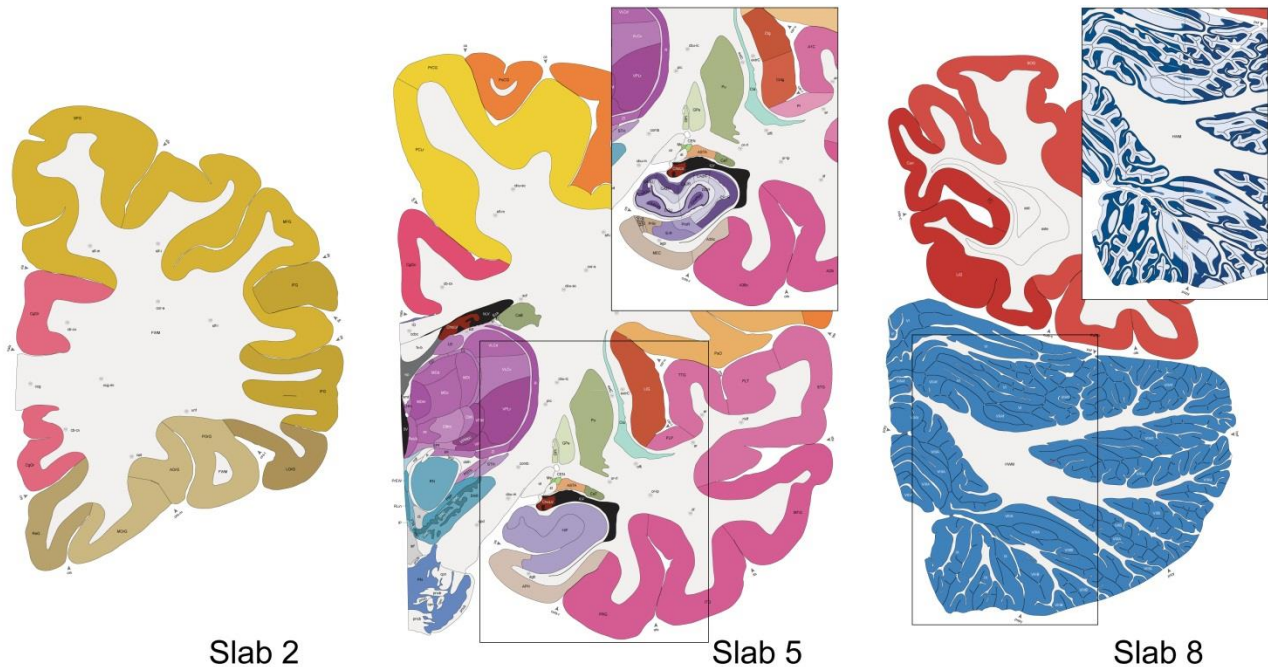


Figure 8. Reference atlas plates from the adult reference atlas. Coronal reference atlas plates from slabs 2, 5 and 8 of the adult reference atlas specimen are shown (left hemisphere). Structurally, green represents cerebral nuclei, including amygdala, basal ganglia, and basal nuclei; blue represents hindbrain, including pons, medulla and cerebellum. Purple represents thalamus, sky-blue represents midbrain, teal represents claustrum, and lavender represents allocortex. Light gray and black represent white matter and ventricles, respectively. Lobes and areal regions of the neocortex are also represented by color: mustard and canary for frontal, orange for parietal, rose for temporal, salmon for cingulate, brick for insular, and rouge for occipital. An alternate view of cerebral and cerebellar cortices based on cyto- and chemo-architectonics is also available (slab 5 and slab 8 insets, respectively), as are prominent fiber bundles throughout the forebrain (white matter hotspots, slab 5 inset).

REFERENCES

Karnovsky MJ, Roots L (1964) A "Direct Coloring" Thiocholine Method for Cholinesterases. *J Histochem Cytochem* 12:219-221.