ALLEN INSTITUTE for BRAIN SCIENCE
Fueling Discovery

ALLEN **Mouse Brain Connectivity Atlas**

TECHNICAL WHITE PAPER: REFERENCE DATASET

OVERVIEW

Mouse brain regions can be delineated by cytoarchitecture, myeloarchitecture and chemoarchitecture like other mammalian brains with a variety of histological methods. These methods combined together provide neuroanatomic detail far beyond what can be achieved by Nissl staining or other counterstaining methods alone. Therefore, diverse, reference datasets derived from various histological and immunohistochemical stains are provided in the Allen Mouse Brain Connectivity Atlas (the Atlas). Neuroimaging, such as MRI or DTI, or additional histological staining methods may be added in the future. The reference data will help enable (1) more precise annotation of the connectivity data, (2) annotation of fiber tracts, and potentially (3) definition of anatomic structures based on the new connectivity information in addition to what the current mouse atlases describe.

The Allen Mouse Brain Connectivity Atlas data are based on fixed brain tissue, as described in the Overview white paper. In contrast, the Allen Reference Atlas—developed as part of the Allen Mouse Brain Atlas and now used as the framework onto which the connectivity dataset will be registered—was developed using a Nissl stained image series generated from snap-frozen, unfixed brain tissue. In order to bridge the gap between these two different methodologies to achieve the best registration results, an interface needs to be created for the fixed brain tissue and mapped back to the Allen Reference Atlas. Therefore, new Nissl data were generated using fixed mouse brain tissue, not only to provide cytoarchitectural information, but also to aid in the registration process.

An array of histological staining methods was selected to generate reference data for the Atlas, all of which were considered for their (1) uniqueness of cytoarchitectonic contents and (2) feasibility in fixed brain tissue. Specifically, two histological staining methods, including Nissl, and immunostaining with 6 different antibodies were chosen and put into combinations yielding five final image series as shown in Table 1 below.

Table 1. Histological staining methods for the reference data

Staining Type	Image series	Stain	Target	Staining Pattern
Histological staining (colorimetric)	1	Nissl	Nissl substances	Pan-cellular, cell bodies
	2	AchE	Synaptic protein Acetylcholinesterase	Cholinergic transmission
Immunohistochemistry (fluorescent)	3	Pvalb	Ca^{2+} binding protein Parvalbumin	Cell-type marker: a subset of inhibitory and excitatory neurons
		SMI-32	Neurofilament-H (non-phosphorylated)	Cell bodies, dendrites and thick axons

	4	Calb1	Ca^{2+} binding protein Calbindin, 28 kDa	Cell-type marker: a subset of inhibitory and excitatory neurons
		SMI-99	Myelin basic protein	Myelinated axons, oligodendrocytes
	5	NeuN	Nuclear protein Fox3	Cell-type marker: Pan-neuronal
		NF-160	Neurofilament-M	Cell bodies, dendrites and axons

The Nissl staining technique is commonly used to study the cytoarchitectonics of different brain areas (Nissl, 1894). The Nissl staining method is based on the interaction of basic dyes such as cresyl violet or thionin with the nucleic acid content of cells. These dyes intensely stain "Nissl substances" (rough endoplasmic reticulum) which are abundant in neurons, and reveal specific patterns of cytoarchitecture in the brain, such as the laminar pattern of the cerebral cortex or the interlocking nuclear patterns of the diencephalon. Acetylcholinesterase (AChE) participates in cholinergic transmission by enzymatic degradation of a synaptic transmitter, acetylcholine. AchE staining has been found in both axons and cell bodies, and provides unique cytoarchitectonic patterns in various cortical and subcortical areas (Karnovsky and Roots, 1964).

The chemoarchitectures are shown with various antibody stainings exhibiting unique distribution of cell types or cellular processes. Anti-SMI-32 antibody reacts with a nonphosphorylated neurofilament H (Sternberger *et al.* 1983), and can be used to visualize neuronal cell bodies, dendrites and thick axons in the central nervous system. Anti-NF160 antibody reacts with both phosphorylated and non-phosphorylated forms of medium neurofilament protein (160 kDa) of various species (Halfter et al., 1994). Both anti-SMI-32 and anti-NF-160 antibodies can be used to delineate many cortical regions as well as subcortical structures. Anti-SMI-99 antibody reacts with myelin basic protein (Dowse et al., 1983) and is used to visualize myelinated axon fibers and oligodendrocytes.

Two calcium-binding proteins, parvalbumin (Pvalb) and calbindin1 (Calb1), mark subsets of inhibitory and excitatory neurons (Celio et al., 1981; Celio, 1990). Both Pvalb and Calb1 have been widely used to provide chemoarchitecture of many brain regions (Paxinos and Watson, 2009). Anti-NeuN antibody specifically recognizes the DNA-binding, neuron-specific protein NeuN, which is present in most neuronal cell types, and thus serves as a pan-neuronal marker (Mullen et al., 1992). The NeuN protein is predominantly found in nuclei and has recently been identified as the nuclear protein Fox3.

SPECIMEN PREPARATION

Mice were anesthetized with 5% isoflurane and intracardially perfused with 10 ml of saline (0.9% NaCl) followed by 50 ml of freshly prepared 4% paraformaldehyde (PFA) at a flow rate of 9 ml/min. Brains were rapidly dissected and post-fixed in 4% PFA at room temperature for 3-6 hours and overnight at 4°C. The next day, brains were transferred to a 30% sucrose solution and left for approximately 2 days until they sunk to the bottom of the tube. Cryoprotection in sucrose has been shown to protect brains from ice crystal formation during the cryosectioning step. Brains were embedded in OCT and stored at -80°C prior to sectioning as previously described.

TAPE TRANSFER SECTIONING

The perfused brains were sectioned at 25 µm on a Leica 3050 S Cryostat equipped with an Instrumedics Tape Transfer System that includes a UV light polymerization chamber and warming pad. A roll of Instrumedics tape flags (Instrumedics cat. #TW) was cut into individual flags. Slides were coated with 50 µL of Solution B adhesive (Instrumedics cat. #475272) and allowed to air dry. Prior to taking a section, slides were positioned on the warming pad. The tacky surface of the tape flag was positioned over the block face of the tissue. Sections were cut and placed on the slide on the warming pad. A brush was used to apply pressure to

the tape flag so that it was thoroughly laminated to the slide. After all tape flags for a slide were laminated, the slide was placed into the UV light polymerization unit. Following UV light treatment, the tape flags were removed with forceps, leaving the sections attached to the slide. Alternating sections were collected to give a final sampling density of 50 µm. After brain tissue was sectioned, slides were kept at room temperature for two hours, boxed and stored at -80°C until used for staining.

COLORIMETRIC STAINING

Nissl Staining

Slides were removed from the freezer and allowed to equilibrate to room temperature. Sections were delipidated with the xylene substitute Formula 83 (CBG Biotech cat. #CH0104), and hydrated through a graded series containing 100%, 95%, 70% and 50% ethanol. After several washes in water, the sections were stained in 0.21% thionin for 3 min, then dehydrated in water and a graded series containing 50%, 70%, 95% and 100% ethanol. Slides were differentiated during the 95% EtOH step of dehydration, using 0.1% glacial acetic acid in 95% ethanol. Slides were monitored during this step and remained submerged in the solution until they were sufficiently differentiated, before a final dehydration step using 100% ethanol. Finally, the slides were incubated in Formula 83, and coverslipped with the Curemount mounting medium (Instrumedics cat. #475232). After drying, the slides were analyzed microscopically to ensure staining quality. Slides that passed QC were imaged using a ScanScope scanner (Aperio Technologies, Inc, Vista, CA).

Acetylcholinesterase (AChE)

Slides were removed from the freezer and allowed to equilibrate to room temperature. Sections were fixed in 10% neutral buffered formalin (NBF) and washed briefly in ultra pure water. Sections were then incubated for 25 minutes in the incubation solution (0.5 mg/ml acetylthiocholine iodide, sodium citrate, cupric sulfate, and potassium ferricyanide in a 0.1M sodium acetate buffer (pH 8.0)), 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 rinsed in water, dehydrated, cleared with Formula 83 and coverslipped with Curemount. The slides were imaged on ScanScope scanner.

IMMUNOHISTOCHEMISTRY

The slides for image series 3-5 (Table 1) were treated with a series of reagents as summarized below and in Table 2 and 3 to allow fluorescent labeling with each antibody. After completion of staining, the slides were counterstained with DAPI and coverslipped with Fluoromount-G medium. The slides were imaged on the VS110/VS120 scanner.

Reagents used

Antigen Retrieval: 10 mM sodium citrate (pH 6.0)
Washing solution: 1x PBS
Blocking solution: 4% normal goat serum (Vector Lab cat. #S-2000) + 0.3% Triton X-100 in PBS
Normal Goat Serum (Vector S-1000 or equivalent)
Primary antibodies:

- Calb1-*Rabbit* (Swant, cat. #CB 38)
- Pvalb-*Rabbit* (Swant, cat. # PV25)
- SMI-32-*Mouse* (Covance, cat. #SMI-32R)
- SMI-99-*Mouse* (Covance, cat. #SMI-99P)
- Neu-N-*Mouse* (Millipore, cat. #MAB377)
- 160 kD Neurofilament Medium antibody (NF-160)-*Rabbit* (Abcam Cat. # ab9034)

Secondary Antibodies:

- Goat Anti-Mouse-488 IgG antibody (Invitrogen, cat. #A-11001)
- Goat Anti-Mouse-594 IgG antibody (Invitrogen, cat. #A-11005)
- Goat Anti-Rabbit-488 IgG antibody (Invitrogen, cat. #A-11008)
- Goat Anti-Rabbit-594 IgG antibody (Invitrogen, cat. #A-11012)

DAPI staining solution (Invitrogen cat. # D1306): final conc. of 200 ng/ml
Fluoromount G (Southern Biotech cat. #0100-01)

Table 2. Immunohistochemistry procedures.

Step	Procedure
Antigen Retrieval	20 min at 95°C
Washing	3 x 5 min
Blocking	1 hr
Primary antibody	Overnight
Washing	3 x 2 hr *
Secondary antibody	Overnight
Washing	3 x 2 hr *
Counterstaining	15 min, followed by several PBS washes

* Extensive washes (3 x 2 hr) are not absolutely required but chosen to suit the workflow for overnight incubation. All the staining procedures were done at room temperature unless noted otherwise.

Table 3. Antibody concentrations used for immunohistochemistry.

	Image Series #3		Image Series #4		Image Series #5	
Primary antibody	Pvalb-R	SMI-32-M	Calb1-R	SMI-99-M	NF-160-R	Neu-N-M
	1:2000	1:1000	1:2000	1:1000	1:1000	1:1000
Secondary antibody (anti-Rabbit-488)	1:1000		1:1000		1:500	
Secondary antibody (anti-Mouse-594)		1:500		1:500		1:500

Image Acquisition

Slides for image series 1-2 (colorimetric) were scanned on a ScanScope scanner (Aperio Technologies Inc.). Slides for image series 3-5 (fluorescent) were scanned on a fully automated, high-speed multichannel fluorescence scanning system VS110 or VS120 (Olympus, Center Valley, PA). Both imaging systems were equipped with 10x objectives.

Data Processing

Once images were acquired, the Informatics Data Pipeline (IDP) managed image preprocessing, image QC, annotation QC and public display of information via the Web application. The IDP has been described in detail previously (Dang *et al.,* 2007), and has been extended to process images for this project. In the preprocessing step, bright field images are white-balanced and a tissue detection algorithm assigns bounding boxes to individual tissue sections, which are manually assessed and adjusted when necessary. For dark field images, the intensity of each color channel has been transformed (window/level) to enable better visualization of the information content. The same transform parameters have been applied to all images. This is followed by tissue detection to generate bounding boxes.

Quality Control

Prior to public release of the data, quality control (QC) is implemented for the reference data similar to other data modalities. In general, all image series are inspected for artifacts which may reduce consistency, ability to analyze, or completeness of the data, and any section exhibiting these artifacts is not released on the public web application. Artifacts such as missing tissue or sections, poor orientation, poor staining quality, edge cutoff, tessellation, flipped sections, and low signal strength may cause image series failure.

For reference data, QC takes place in two steps. The first step is to check dorsal-ventral and left-right orientation for accordance with the Allen Reference Atlas. If it is not similarly oriented, then the reference set is failed and rerun. Once orientation QC is passed, the image series is allowed to enter the staining process. The second QC step is a section-by-section check for artifacts such as poor staining or sectioning quality, and any section exhibiting these artifacts is not released on the public web application. A reference dataset will be failed if there is a gap of more than 100 µm between passed sections. If an image series is failed at the second QC step it will be rerun. Only image series passing both QC steps are incorporated into the Atlas and released to the public.

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