NIH Blueprint NHP Atlas

TECHNICAL WHITE PAPER:
MRI AND HISTOLOGICAL REFERENCE SERIES OF POSTNATAL BRAIN DEVELOPMENT IN RHESUS MACAQUE

OVERVIEW

Non-human primates provide experimentally tractable model systems that allow carefully controlled analysis of important developmental processes relevant for human health and disease. The goal of the NIH Blueprint Non-Human Primate (NHP) Atlas is to provide a detailed analysis of gene expression in the normal developing primate brain in brain regions associated with high-level cognitive, mnemonic and emotional functions as well as neurodevelopmental disorders linked to disruption of these capacities.

A major component of the NHP Atlas is genome-wide transcriptional profiling aimed at the identification of transcriptional programs differentially active at different stages of brain maturation in neonates, infants, juveniles and adults. Also, a significant component of the NHP Atlas consists of cellular level *in situ* hybridization (ISH) on medial prefrontal cortex, primary visual cortex, hippocampus, amygdala, and ventral striatum with a panel of genes for each structure assayed across four time points (birth, 3 months, 12 months, and 48 months). In addition, ISH data serially across complete hemispheres of three 48-month male specimens was included. To complement these postnatal gene expression data sets, stage-specific magnetic resonance imaging (MRI) and histological reference series for each developmental time point was generated to provide a developmental neuroanatomical framework of reference for the ISH and microarray data.

An interleaved atlas of MRI, histology, and block face photographs was created from four male rhesus macaques at the following ages: 2 weeks (birth), 3 months, 12 months, and 48 months. The protocol to generate this data consisted of several components: high resolution MRI, transcardial perfusion, rod insertion, embedding, cryoprotection, flash freezing, attaching the embedded brain to the microtome stage, sectioning, mounting, and staining, which were all performed at University of California Davis. Image acquisition and image processing were completed at the Allen Institute.

TISSUE SPECIMENS AND IMAGING

Tissue samples from male rhesus macaque (Macaca Mulatta) were obtained through the California National Primate Research Center (CNPRC; http://www.cnprc.ucdavis.edu/). Tissue samples were systematically collected from well-characterized rhesus monkeys born and raised in outdoor, ½-acre naturalistic enclosures located at the CNPRC, which provide a naturalistic setting and normal social environment. Extensive health, family lineage and dominance information is maintained on all animals in the outdoor enclosures. All procedures were approved by University of California Davis IACUC.

Male rhesus macaques were scanned at the University of California Davis Imaging Research Center (IRC) using a 1.5 Tesla GE Genesis Signa MRI scanner. Animals were removed from field cages the evening prior to scanning, or the morning of scanning and then transported to Sacramento by California National Primate Research Center (CNPRC) staff. Veterinarians and Animal Heath Technicians (AHTs) accompanied the animals if they were younger than one month. Once the rhesus monkeys arrived at the IRC, the animals were sedated by injection of Ketamine. The animals were then intubated and an IV catheter was inserted. MRI compatible infusion pumps were used to administer a Propofol and saline solution as anesthesia. Once the rhesus monkeys were completely anesthetized, they were taken into the scanner room and placed into an MRI compatible stereotaxic apparatus.

The stereotaxic apparatus was leveled and aligned in the scanner using the laser alignment system. The laser was precisely at AP 0 on the stereotaxic apparatus and the centerline laser ran along the center of the eye-bar/palate-bar plate. The ear-bar and palate plate numbers were recorded and used when the animal was replaced in the stereotaxic apparatus during the brain extraction. For 2 week and 3 month animals, a 3-inch circular coil was mounted to the stereotaxic apparatus, centered, and leveled over the head of the

animal. For 12 month and 48 month animals, the stereotaxic apparatus and the animal were placed into a human head coil.

T1 weighed high-resolution structural SPGR scans were acquired as follows:

For 2 week and 3 month animals:

SPGR_ T1 weighted
Orientation: Coronal
FOV: 80mm X 80mm
Image size: 256x256
Thickness: 1mm
Spacing: 0
TE: 7.9
TR: 21
FS: 15000
NEX: 4
Pixel size: 0.3125mm

For 12 month and 48 month animals:

SPGR _ T1 weighted
Orientation: Coronal
FOV: 160mm X 160mm
Image size: 256x256
Thickness: 1mm
Spacing: 0
TE: 7.9
TR: 21
FS: 15000
NEX: 4
Pixel size: 0.625mm

After completion and verification of the scan, the animal was removed from the stereotaxic apparatus and from monitoring and anesthesia devices, and was then transported back to the CNPRC for perfusion. MR images were filtered and normalized. The MR images were used to calculate the insertion of reference posts into the brain.

TISSUE PROCESSING

For transcardial perfusion, all solutions were made the morning of the perfusion to allow time for cooling to 4°C. One percent paraformaldehyde in 0.1 M sodium phosphate buffer was run through a peristaltic pump at a rate of 250 ml/min for 2 minutes for animals > 1 kg. Animals < 1 kg were perfused at a rate of 100 ml/min for 2 minutes. Following the 1% paraformaldehyde, 4% paraformaldehyde in 0.1 M sodium phosphate buffer was perfused at a rate of 250 ml/min for 10 minutes then slowed to a rate of 100 ml/min for 50 minutes for animals that were > 1 kg. For animals < 1 kg, the rate was 100 ml/ min for 60 minutes. When the perfusion was done, the skin and the skullcap were removed, and the dura was retracted. The skull was placed into the stereotaxic apparatus that was used for the MRI, ensuring that the MRI was appropriately aligned with the brain position during sectioning for histology.

Using the structural MRI, the appropriate positioning for the rod insertion was determined. Metal rods were approximately 1 mm in diameter. A sharpened stylus ran through the rods to help penetration into the brain. A stereotaxic tower was used to maintain the orientation of the marker rods perpendicular to the stereotaxic plane. Rods were inserted into the brain according to calculations from the MRI and inserted until the stylus reached the base of the skull. The stylus was removed and then the rods from the holders that were attached to the towers were separated. The brain was removed from the skull while leaving it in the stereotaxic

apparatus and gently lifting on the frontal poles (see **Figure 1**). Periostal elevators were used to cut the 12 cranial nerves and extract the brain. The brain was then post-fixed in 4% paraformaldehyde overnight. The rods remained in the brain until it was ready for sectioning.

For embedding, the next day the brain was lowered into a rectangular mold containing molten gelatin (10% gelatin 275 Bloom in water). The mold was placed onto the base of the stereotaxic apparatus and the posts were held in place by the towers, which kept the appropriate plane for attaching the gelatin to the stage. Then, the entire stereotaxic apparatus, towers, mold, and brain were placed at 4°C. After 6 hours the gelatin was hard enough to extract from the rectangular mold. Being careful not to remove the posts, a spare rod was used to separate the edges of the gelatin from the mold, and the gelatin/brain complex was carefully pulled out. A fine wire was used to trim the excess gelatin into a block that surrounded the brain. The embedded brain was immersed into 4% paraformaldehyde in 0.1 M sodium phosphate buffer at 4°C for 48 hours.

For cryoprotection, the embedded brain was placed in 10% glycerol, 2% dimethylsulfoxide (DMSO) in 0.1 M sodium phosphate buffer for 7 days. Then, the brain was then transferred into 20% glycerol, 2% DMSO in 0.1 M sodium phosphate buffer, with 250 mg/L of paraformaldehyde (to inhibit yeast and fungal growth) for 2 months.

For flash freezing, a beaker of 2-methyl-butane (isopentane) was immersed in a 100% ethanol bath, and then chilled by adding crushed dry ice to the ethanol. Strings were tied to the posts in the gelatin block, and were used to suspend the embedded brain in the bath. The brain was frozen for 1 hour (checked frequently to make sure that the dry ice bath was full of dry ice). The block was wrapped in aluminum foil, labeled, and stored at -80°C.

To attach the embedded brain to the microtome stage, Plexiglas® rectangles were used to form a container around the inner portion of the stage. The posts that protruded through the gelatin were used to rest on the Plexiglas forms, which maintained the appropriate angle for cutting. Cryo-gel (Instrumedics CAT# 475237) was poured onto the stage inside the rectangular forms. Dry ice was added to the outer ring of the stage, as the Cryo-gel on the bottom began to freeze, the brain/gelatin block was placed with protruding posts onto the forms on the stage. The block was checked to make sure it was not floating and that there was enough Cryo-gel to contact the base of the block. Dry ice was added around the stage until the Cryo-Gel was frozen. Then, Plexiglas forms were carefully removed. The stage was used to align the gelatin mold in the appropriate position to drill perpendicular to the angle of cutting. Holes were drilled into the gelatin 50 mm apart on center. These holes were then used to align the section with the corners of the microscope slides. An ice dam was placed in position and filled with dry ice. The plastic base of the stage was marked where it meets the chuck, this ensured that the stage was replaced in the same position every time cutting was initiated. The camera was aligned and the microtome was locked down in position with c-clamps.

Sections were cut with a high profile blade (Extremus CAT# D554XD) in a Leica blade holder that was set at 2.5°. Sections were 50 µm thick, in a 1:5 series with the following order: series 1 was in phosphate buffer for immediate mounting and Nissl staining; series 2 was in 10% formalin for back-up Nissl; series 3, 4, and 5 were collected in Tissue Collection Solution (TCS) for long-term storage. It was critical that dry ice was maintained around the gelatin block during cutting. A scale bar was photographed with the cut surface at the beginning of each day and after removing any spacers holding the microtome blade. Great care was taken to make slow even sections. Pictures (blockface images) were taken every fifth section.

For mounting the sections, in a petri dish of 0.05 M sodium phosphate buffer the sections were unfolded and flattened. The two holes in the gelatin were hooked over the corner of the slide and the section was carefully lifted out of the solution. Sections were dried flat, or at a slight angle to avoid any distortion. Slides were allowed to dry overnight in a warm oven. The next day the slides were defatted in a 1:1 mixture of chloroform and ethanol. After which the sections were partially hydrated and dried again in the warm oven. The next day slides were stained with Nissl.

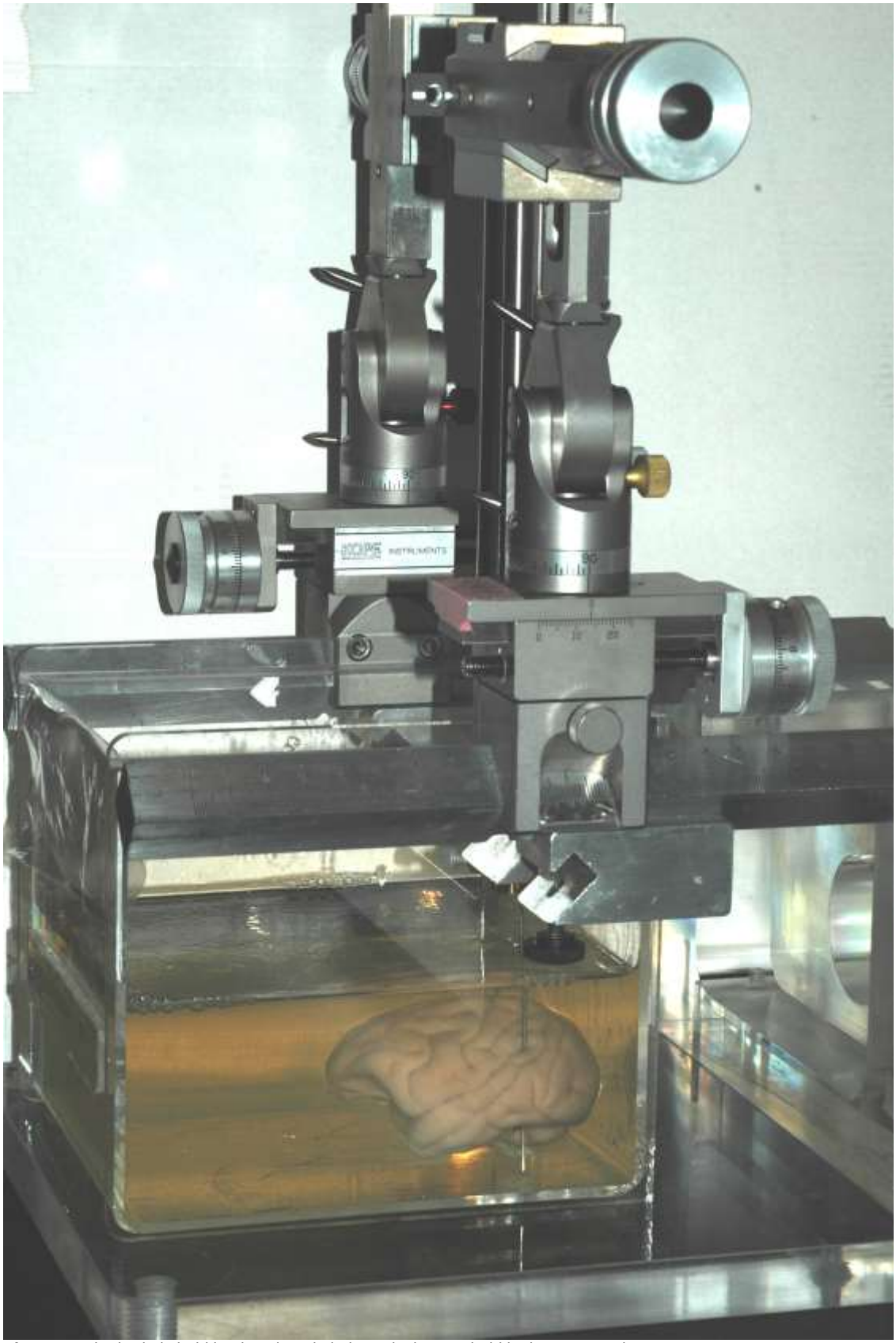

Figure 1. The brain is held in place in gelatin by rods that are held in the stereotaxic tower.

NISSL STAINING

After chloroform:ethanol treatment for a total of 4 hours (2 hours in chloroform:ethanol then 2 hours of fresh chloroform ethanol), sections were partially hydrated in 100% ethanol, 100% ethanol, and 95% ethanol for 3 minutes at each ethanol concentration. Then, sections were air dried for 10 minutes and placed into a 37°C warming oven overnight. The following day slides were dipped into de-ionized water then placed directly into 0.25% Thionin stain for 30 seconds. Once the slides were stained, they were dipped for several seconds into two consecutive de-ionized water dishes followed by dehydration. The dehydration process consisted of incubation in 50% ethanol, 70% ethanol, 95% ethanol with 5-7 drops of glacial acetic acid, 95% ethanol, 100% ethanol, and 100% ethanol for 4 minutes each incubation. After dehydration, sections were put through 3 successive xylenes for four minutes each. The slides were coverslipped with DPX and placed flat on drying trays, covered for at least three weeks at room temperature before slides were placed in slide boxes. Slides were stored at room temperature in slide boxes before being transferred to the Allen Institute for cleaning, image acquisition, and data processing.

IMAGE ACQUISITION

Image acquisition was performed using ScanScope® scanners (Aperio Technologies, Inc.; Vista, CA). The line scan camera continually adjusts for focus based on a variable number of focus points and provides advantages over tile-based image acquisition platforms for large tissue sections that tend to have more variation in height. The ScanScope scanner uses a 20x objective that is downsampled in software to minimize acquired data volume. The downsampling provides an image resolution of approximately 1.00 µm/pixel.

DATA PROCESSING

Once images were acquired, the Informatics Data Pipeline (IDP) managed image preprocessing, image quality control (QC), Nissl processing, annotation QC and public display of information via the Web application.