ALLEN INSTITUTE for BRAIN SCIENCE
Fueling Discovery

ALLEN **Mouse Brain Connectivity Atlas**

TECHNICAL WHITE PAPER:
BDA VS. rAAV COMPARISON

OVERVIEW

Circuit mapping has historically been performed using non-viral retrograde or anterograde tracers. However, the recombinant adeno-associated virus (rAAV) has been widely adopted recently for labeling axonal projections because of its distinct advantages over classic anterograde tracers such as biotinylated dextran amines (BDA) or phytohemagglutinin (PHA-L) (Chamberlin *et al.*, 1998; Betley and Sternson, 2011). In spite of the increasing use of viral tracers, little data exists to allow one to compare labeling efficiency or specificity between two popular tracing methods in diverse brain regions.

In order to compare viral and conventional (BDA) tracers, approximately 20 brain regions were selected throughout the brain to represent broad anatomical areas and diverse cell types. rAAV and BDA were co-injected into each selected brain region in wild-type mice using a sequential injection method developed to target virtually the same anatomic region. For most cases presented, the anatomical area(s) of tracer uptake are well matched. However, there are cases with considerable differences in the uptake area between BDA and rAAV due to uneven infection or leakage along the needle tract or into neighboring anatomical regions. These data should be interpreted with this caveat in mind. Axonal projections from neurons infected/labeled by rAAV or BDA were visualized with distinct fluorescent colors in subsequent histological staining procedures and imaged on an automated fluorescence scanner.

To control for experimental variation and obtain an interpretable dataset with high accuracy, a total of N=4 replicates will be generated for each of the 20 brain regions.

Brain Region Selection

Twenty brain regions were chosen to compare anterograde axonal tracing between BDA and rAAV. These 20 regions were selected based on the following criteria and are listed in Table 1:

1. **Representative**: to represent major brain structures, diverse cell types and connectivity.
2. **Well-established**: rich information on projection targets available from the existing literature.
3. **Accessible**: relatively large area, easy to target, to reduce variability between replicates.

Table 1. Brain regions selected for BDA vs. rAAV comparison.

Basic cell group	Brain region	Reference atlas acronym
Isocortex	Primary motor area Primary somatosensory area, barrel field Primary visual area	MOp SSp-bfd VISp
Cortical Subplate	Basolateral amygdalar nucleus, anterior	BLAa
Hippocampal formation	Field CA1 Subiculum	CA1 SUB
Olfactory areas	Anterior olfactory nucleus	AON
Pallidum	Globus pallidus, external segment	GPe
Striatum	Caudoputamen Lateral septal nucleus	CP LS
Hypothalamus	Lateral hypothalamic area	LHA
Thalamus	Dorsal part of the lateral geniculate complex Ventral posteromedial nucleus of the thalamus	LGd VPM
Midbrain	Inferior colliculus, external nucleus Red nucleus Substantia nigra, reticular part Superior colliculus, sensory related	ICe RN SNr SCs
Cerebellar Cortex	Vermal region lobule 4,5	CUL4,5
Medulla	Inferior olivary complex	IO
Pons	Motor nucleus of trigeminal	V

Mice

Adult male C57BL/6J (stock 00064), B6129PF1/J hybrid (stock 100492) or B6129SF1/J hybrid (stock 101043) mice were purchased from The Jackson Laboratory and acclimated to our facility for at least 5 days prior to surgery. Mice were group housed (5 per cage) in micro ventilated cages with a 12 h light/dark cycle. Purina Lab diet 5001 mouse food and water were given *ad libitum*. All surgeries were done in adult mice at postnatal day (P)56 ± 2.

Anterograde tracers

Biotin dextran amine (BDA-10,000; Invitrogen, Carlsbad, CA) was diluted in PBS for injections. The rAAV vector used for this study was purchased from the Penn Vector Core (University of Pennsylvania, Philadelphia, PA). Briefly, the rAAV vector (serotype 1) expresses enhanced GFP (EGFP) under control of a human synapsin I promoter with the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and bovine growth hormone polyadenylation sequences (rAAV2/1.hSynapsin.EGFP.WPRE.bGH). Methods used for the packaging, purification and determination of titer for the rAAV vector can be found at http://www.med.upenn.edu/gtp/vectorcore/quality_control.shtml.

Selection of stereotaxic coordinates

Stereotaxic coordinates were chosen for each target area based on *The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2001). The anterior/posterior (AP) coordinates are referenced from Bregma. The medial/lateral (ML) coordinates are distance from midline at Bregma, and dorsal/ventral (DV) coordinates are measured from the pial surface of the brain. For primary motor (MOp) and somatosensory cortical areas (SSp-bfd), injections were made at two depths to label neurons throughout all 6 cortical layers. For primary somatosensory (SSp-bfd) and primary visual areas (VISp), the pipette was inserted into the brain

at an angle to infect neurons along the same cortical column. The stereotaxic coordinates used for the BDA vs. rAAV Comparison are listed in the *Injection Sites & Stereotaxic Coordinates* white paper under the Documentation tab.

Sequential stereotaxic injections of rAAV and BDA using iontophoresis

Mice were anesthetized with 5% isoflurane and placed into a stereotaxic frame (Model# 1900, Kopf, Tujunga, CA). The isoflurane level was maintained at 1-5% throughout the surgery. An incision was made to expose the skull, and Bregma and Lamda landmarks were visualized using a stereomicroscope. A hole was made overlying the targeted area by first thinning the skull using a fine drill burr until only a thin layer of bone remained. A microprobe and fine forceps were used to peel away this final layer of bone to reveal the brain surface. Each mouse was injected twice into a single target region. First, a glass pipette (inner tip diameter of 10-20 µm) loaded with virus was lowered to the desired depth from the pial surface of the brain. Currents were applied for iontophoresis of rAAV particles (3 µA, 7 sec on/7 sec off cycle, for 5 min). The pipette was left in place for 5 minutes before withdrawal. Then the same process was repeated at the same location to deliver 10% BDA in a second pipette. The majority of injections were made using 3 µA currents as these settings resulted in infection areas of approximately 400 – 700 µm in diameter in most target regions. For regions where these settings produced larger areas of uptake (e.g. Substantia nigra, reticular part (SNr) and Red nucleus (RN)), the current strength was reduced to 1 µA. Mice were quickly recovered after surgery and survived for 14 days prior to sacrifice.

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 rinsed briefly with PBS. After removing residual moisture on the surface with a Kimwipe, a permanent marker (BIC Mark-It) was applied on the left hemisphere to stain both dorsal and ventral surfaces as shown in Figure 1. Then, the brains were transferred to 30% sucrose solution, and left for approximately 2 days until they sink to the bottom of the tube. Cryoprotection in sucrose has been shown to protect brains from ice crystal formation during the following cryosectioning step. In addition to the marker, the brains were skewered using a 22 gauge needle through the mid-sagittal plane of the left hemisphere. Either the residual ink on the brain surface or the punched hole on the left side of the brain was used to distinguish left vs. right hemispheres during mounting of free floating sections. Brains were embedded and frozen in OCT mounting medium in a grid-lined freezing chamber for standardized placement of brain as previously described (*Data Production Processes* in the Documentation section of the Allen Mouse Brain Atlas). Brains were stored at -80°C prior to sectioning.

Figure 1. Brain is stained with black marker on the left hemisphere to help distinguish left/right during mounting of free floating sections, Views from the dorsal (left panel, anterior side up) and ventral (right panel, anterior side down) surfaces are shown above.

Cryosectioning

The perfused brains were sectioned at 50 µm on Leica 3050 S cryostats. This thickness is optimal for minimizing sectioning artifacts such as stretching and folding during cutting but adequate for reagent penetration into the section during the staining procedure necessary to fully label fine axonal projections. Each OCT block containing a brain was trimmed until reaching the coronal level 132 of the associated reference atlas. Starting at this point, approximately 240 sections spanning the rest of the brain were collected into two alternating series. Therefore, each series maintains 100 µm sampling density between adjacent sections which is sufficient for even small neuro-anatomical structures to be represented in at least one section. The first series was collected directly onto PLUS superfrost slides coated with 0.3% gelatin solution for detection of BDA and rAAV signal. The second series was collected into 24-well plates containing PBS with 0.1% of Sodium Azide for further immunostaining of SMI-32.

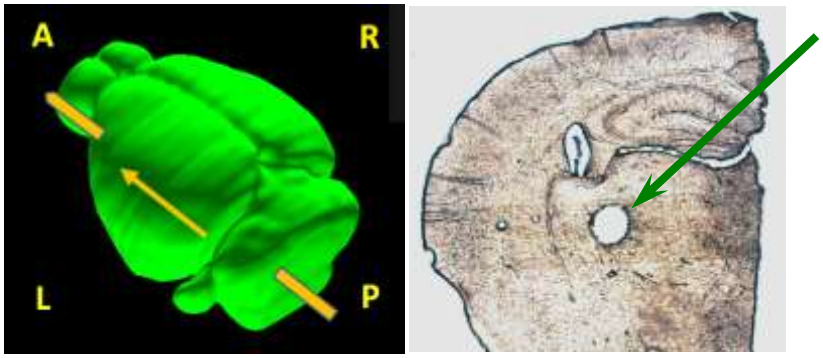

Figure 2. Illustration of skewering method (left panel) and the resultant hole observed on the tissue (right panel).

Immunohistochemistry/Histochemistry

The slides (1st series) were treated with a series of reagents as summarized below and in Table 2 to fluorescently label rAAV-EGFP and BDA. The free floating sections (2nd series) were subject to immunostaining with anti-SMI-32 antibody to be used for better delineation of anatomic boundaries. Although the slide-based staining method has advantages in minimizing section deformation or other tissue damage, the free floating method was chosen for the 2nd series to enhance the penetration of anti-SMI-32 antibody. The slide-based method was chosen for the 1st series since reasonable staining results were observed using anti-GFP antibody and Streptavidin conjugate for detection of rAAV-EGFP and BDA using this method. The immunostained free floating sections were mounted onto slides. The slides from both series were baked at 37°C overnight, counterstained with DAPI and coverslipped with Fluoromount-G medium.

Reagents used:

- Washing buffer: PBS or PBS with 0.0005% Tween.
- Blocking solution: 4% normal goat serum (Vector Lab cat. #S-1000) + 0.3% Triton X-100 in PBS.
- Rabbit anti-GFP primary antibody (Invitrogen cat. #A11122): final dilution to 1:2000 in blocking solution.
- Mouse anti-SMI-32 primary antibody (Covance cat. #SMI-32R): final dilution to 1:1000 in blocking solution.
- Goat anti-rabbit IgG-AF488 secondary antibody conjugate (Invitrogen cat. #A11034, 2mg/ml): final dilution to 1:1000 (2 µg/ml) in blocking solution.
- Goat anti-mouse IgG1 (γ1)-AF594 secondary antibody conjugate (Invitrogen cat. #A21125, 2mg/ml): final dilution to 1:500 (4 µg/ml) in blocking solution.

- Streptavidin-AF555 conjugate (Invitrogen cat. #S32355, 2mg/ml): final dilution to 1:1000 (2 µg/ml) in blocking solution.
- DAPI staining solution (Invitrogen cat # D1306): final concentration of 5 µM in PBS with 0.0005% Tween.
- Fluoromount G (Southern Biotech cat. #0100-01)

Table 2. Immunohistochemistry/histochemistry procedures.

Step	1st series (BDA plus rAAV) *Slide-based*	2nd series (SMI-32 plus rAAV) *Free floating*
Washing	2 x 10 min (PBS + Tween)	2 x 10 min (PBS + Tween)
Blocking	2 hr	2 hr
Primary antibody	Overnight (anti-GFP)	Overnight (anti-SMI-32)
Washing	2 x 10 min (PBS + Tween)	3 x 20 min (PBS + Tween)
Secondary antibody /conjugate cocktail	Overnight (goat anti-rabbit IgG-AF488 + Streptavidin-AF555)	4 hr (goat anti-mouse IgG-AF594)
Washing	3 x 10 min (PBS + Tween) 1 x 10 min (PBS)	2 x 20 min (PBS)
Counterstaining	3 x 5 min, followed by several PBS washes	3 x 5 min, followed by several PBS washes
Staining results:	Green: rAAV (immunostained) Red: BDA Blue: DAPI	Green: rAAV (native fluorescence) Red: SMI-32 Blue: DAPI

* all the staining procedures were done at room temperature.

Image acquisition

Slides for both series were scanned on a fully automated, high-speed multi-channel fluorescence scanning system VS110 or VS120 (Olympus, Center Valley, PA) equipped with a 10x objective (N.A. 0.4). The resulting images have a resolution of approximately 0.64 µm/pixel. For all experimental data, rAAV and BDA are visualized in the green and red channel, respectively.

Data processing

Once images were acquired, the Informatics Data Pipeline (IDP) managed image preprocessing, image quality control (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 study. In the preprocessing step, 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. A tissue detection algorithm assigns bounding boxes to individual tissue section, which are manually assessed and adjusted when necessary.

In the next step of the pipeline, each data-set is registered to a 3-D reference model. This reference model is built upon the same coronal plates as the reference atlas for the Allen Mouse Brain Atlas. Transforms (12 parameters affine) from the registration process is stored in the database and is used in the web application to support the image synchronization function in the Image Viewer.

Quality control

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

Injection site annotation and polygon drawing

Once an image series passes the quality control step, the data analysis team annotates the anatomical location of injection site(s) based on the reference atlas for the Allen Mouse Brain Atlas and *The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2001). The series immunostained with anti-SMI-32 antibody is also used to guide annotation of injection sites. A representative section containing the injection site is selected and superimposed on the closest reference atlas plane, as exemplified in Figure 3. Ideally, injection sites are confined to a single structure, but in some cases, injections hit multiple structures. If an injection has hit multiple structures, the structure containing the majority of the tracer is named the primary injection site, and any other structures containing tracer are considered secondary injection sites. The primary and secondary injection sites will be provided as part of the public data release. Manually drawn polygons for each passed injection are delineated with an electronic ROI for ease of injection site location by the end user, and further informatics processing.

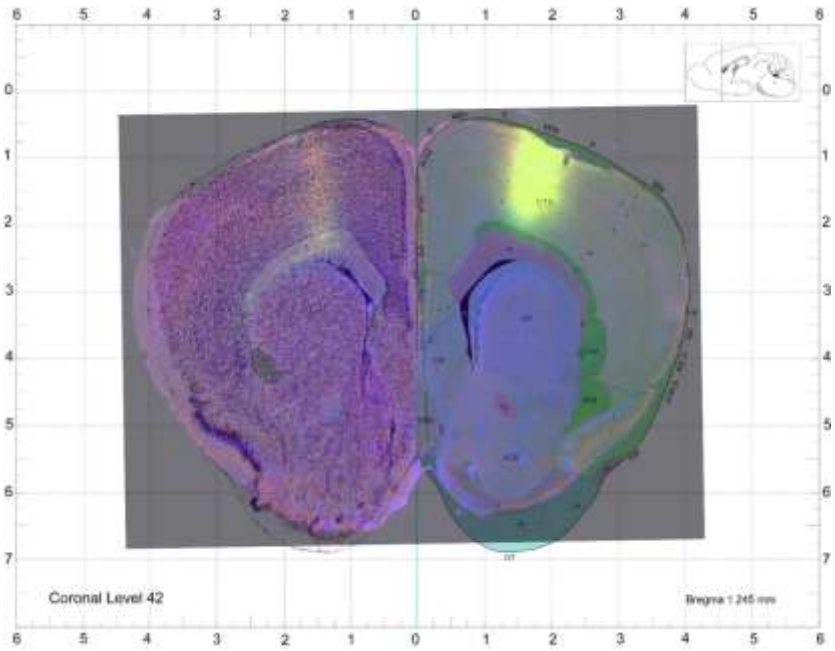

Figure 3. Example of injection site annotation. The scanned image containing the center of the injection site (MOp) was overlaid on the corresponding plane of the reference atlas to aid in determining the injection site structure.

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