ALLEN INSTITUTE for BRAIN SCIENCE
Fueling Discovery

ALLEN **Mouse Brain Connectivity Atlas**

TECHNICAL WHITE PAPER: OVERVIEW

OVERVIEW

The Allen Mouse Brain Connectivity Atlas is designed as a three-dimensional, high-resolution map of neural connections in the mouse brain. Axonal projections from a broad range of brain regions will be mapped on a standardized platform to generate a comprehensive database of neural projections. Built upon an array of transgenic mice genetically engineered to target specific cell types, the Allen Mouse Brain Connectivity Atlas (the Atlas) comprises a unique compendium of projections from selected neuronal populations throughout the brain. By adopting genetic tracing approaches, the Atlas also offers a reproducible framework to facilitate efforts in probing neural circuits and investigating various neural disorders. In addition, the neural connectivity information complements the Allen Institute's gene expression atlases and thus provides opportunities to help understand how genes contribute to connectivity formation and function.

The Atlas is publicly available online via the Allen Brain Atlas data portal (www.brain-map.org) and fully integrated with the other Allen Brain Atlas resources. In addition to the connectivity data, further information about the transgenic mouse lines and genetic tracers used to create the Atlas is also available, thereby allowing other researchers to expand upon the neural connectivity framework from the Atlas.

The Atlas consists of high resolution 2-D projectivity image data that can be viewed side-by-side with associated reference datasets. Tools to enable 3-D visualization and spatial/ontological search of connectivity models through a combination of manual and informatics analyses are also available. Upon completion the Atlas is expected to include:

- **Datasets**
  - Projection Mapping: Axonal projections mapped from ~300 anatomical regions and diverse neuronal populations defined by ~100 different Cre driver lines, labeled by viral tracers and visualized using serial two-photon tomography.
  - BDA vs. rAAV Comparison: Axonal projections from 20 representative brain regions labeled by both conventional and viral tracers and imaged using an epifluorescence microscope for direct comparison of the two tracing methods.
  - Transgenic Characterization: Transgene expression pattern of ~100 Cre and other driver lines characterized in adult and several developmental time points by colorimetric *in situ* hybridization (CISH), fluorescence *in situ* hybridization (FISH) or other histological methods.
  - Anatomic Reference: A collection of histology data and whole-brain neuroimaging data to better delineate cytoarchitectural details, including fiber tracts.
- **Key features**
  - Annotation of the injection sites for all tracer injected brains.
  - Development of a new registration interface from perfused mouse brain and registration into the associated 3-D reference atlas.

- Signal detection, quantification or other informatics analyses of axonal projections.
- 2-D and 3-D interactive, relational database incorporating connectivity information from all brain regions and Cre mice.
- Search and visualization tools for exploring the connectivity data.

Currently, the following data are available on the Allen Brain Atlas data portal (www.brain-map.org):

- Projection mapping from ~200 brain regions including images from multiple Cre driver lines.
- BDA vs. rAAV comparison of more than 20 brain regions.
- 5 series of histological reference datasets.
- Characterization data from more than 100 Cre or other driver lines.

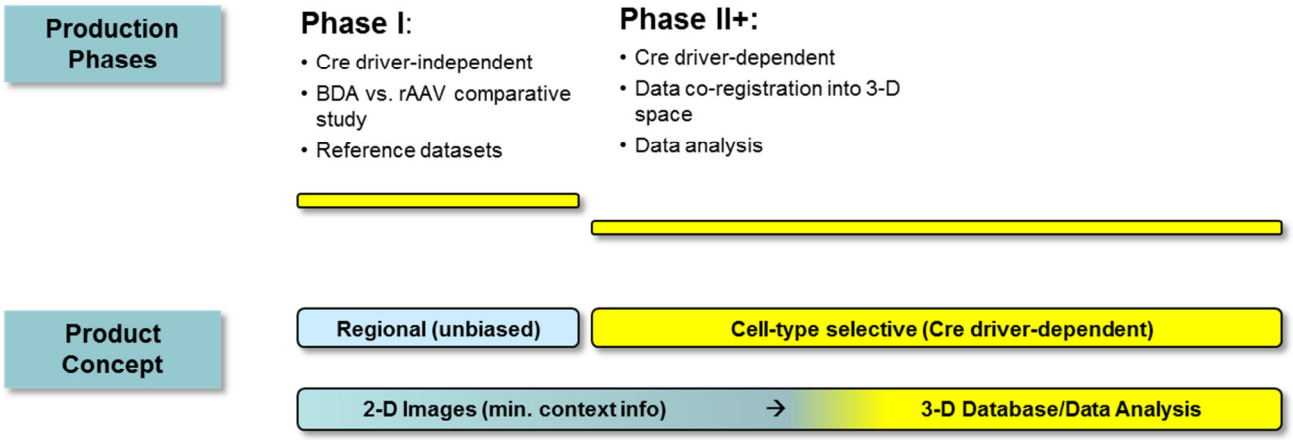

Figure 1. Phases of the Allen Mouse Brain Connectivity Atlas project.
Phase I emphasizes the characterization of axonal projections of wild-type mice. Moving forward, the projection mapping will be expanded to include up to 100 different Cre lines . The relational database will be generated based on the 2-D mapping data which will enable further data analyses in the future.

TRACING METHOD

Advantages of Recombinant Adeno-associated Virus (rAAV) Tracer

Circuit mapping has historically been performed using non-viral anterograde or retrograde tracers. However, labeling axons with viral tracers, such as recombinant adeno-associated virus (rAAV), mediating expression of fluorescent proteins provides distinct advantages over classic anterograde tracers such as biotinylated dextran amines (BDA) or phytohemagglutinin (PHA-L). First, rAAV-mediated expression of fluorescent proteins in infected neurons results in completely labeled cell bodies and axons (Chamberlin et al., 1998). The ability to clearly delineate infected neurons allows one to precisely quantify infection sites and analyze which neuronal types contribute to axonal projections. A second benefit is that rAAV-mediated gene expression is robust and long lasting; transgene expression can be observed months to years after primary infections in the central nervous system (CNS) (Xiao et al., 1997; Charberlin et al., 1998; Hadaczek et al., 2010). This feature makes rAAV particularly useful for visualizing very long axonal projections, which is difficult when using other tracers as they begin to degrade after a short period of time. Also, rAAV-mediated cell labeling produces robust native fluorescence that can be analyzed with no additional histochemical detection steps. This may be desirable for dynamic or in situ imaging, e.g. (Bennett et al., 1997; Stettler et al., 2006; Mittmann et al., 2011). Finally, perhaps the greatest advantage of using rAAVs over traditional anterograde tracers is the flexible molecular strategies that can be used to introduce various transgenes. This enables labeling of selected neuronal populations, e.g. by combining driver mice that express Cre recombinase under the control of a promoter that displays an interesting expression pattern with a rAAV harboring a Cre-dependent expression

cassette. rAAV tract tracing techniques can also be combined with viral-mediated expression of various genetic tool sets, e.g. channelrhodopsins, to allow functional control of neuronal activity within a network of interest. The recent development and use of intersectional genetic strategies has enabled true precision for cell-type specific axonal mapping (Gautron et al., 2010; Kravitz et al., 2010). Therefore, we chose rAAV as the primary anterograde tracer to map projections from diverse brain regions in various transgenic (Cre driver lines) or non-transgenic mice in this study.

Experimental Considerations

Currently, there are a few important factors that affect the utility of rAAV in tracing experiments. Variability in infection is the most commonly encountered issue when using rAAV as an anterograde tracer. This is due in large part to the underlying mechanisms by which viral particles enter the host cell. Normally, viral entry is mediated by the recognition of cell surface receptors (Wu et al., 2006; Van Vliet et al., 2008). The different affinities of rAAV serotypes for specific cell types are influenced by differences in the capsid proteins that recognize these host cell surface proteins. Each serotype has a unique tropism across different cell types. There are many rAAV serotypes now documented; at least 6 of which are commonly used to infect different types of neurons in the CNS to varying degrees (Passini et al., 2003; Burger et al., 2004; Cearley and Wolfe, 2007; Taymans et al., 2007; Cearley et al., 2008). Among these, serotype 1 (rAAV2/1; rAAV2 transfer vector is cross packaged with serotype 1 capsid) appears to have the broadest tropism. We also find that serotype 1 indeed infects neurons in the majority of brain regions we have tested (>250). There may still be better serotypes for particular types of neurons that can be determined empirically and will be employed when necessary to supplement the rAAV2/1 vector used in this study. In addition, we have found that iontophoresis of virus greatly reduces the variability of infection success over multiple experiments, unlike the high amount of variability that can occur with other methods (e.g. pressure injection) (Harris et al., 2012). It is unclear at this point if the mechanisms of viral entry into neurons are affected by iontophoresis, which uses a current to drive electrically charged particles out of the pipette.

An additional consideration when using viral vectors is the survival time required to achieve sufficient gene expression. We obtained results as early as 2-3 weeks after infection sufficient for infected neurons to be brightly labeled in both cell bodies and, more importantly for tracing purposes, their axons in bundled tracts and terminal fields. Single fibers can be detected using native fluorescence at this survival time using various imaging methods capable of high resolution.

Many primarily anterograde tracers also result in retrograde transport to some degree. rAAV is no exception, although levels are generally negligible (Chamberlin et al., 1998). Retrograde infection can occur through active transport of the viral genome from synaptic terminals in the area of injection. The amount of retrograde labeling may depend on brain area, cell types, rAAV serotype or viral preparation (Burger et al., 2004; Cearley and Wolfe, 2007; Taymans et al., 2007; Cearley et al., 2008). We have rarely seen retrogradely infected cells using rAAV2/1 and the methods described here for the majority of brain areas; notable exceptions include some hippocampal subregions and the globus pallidus.

BDA vs. AAV Comparison

To further address the efficiency and reproducibility of rAAV-mediated tracing methods, a focused set of experiments was designed to compare tracing results from a conventional tracer (BDA) with rAAV. The experimental details are described in the *BDA vs. rAAV* white paper under the Documentation tab, and the data can be found under the BDA/AAV Comparison tab in the Atlas.

BRAIN REGION SELECTION

Mapping axonal projections in the adult mouse brain by two-photon imaging of fluorescently labeled neurons have been carried out in two distinct phases, with the first phase emphasizing the characterization of axonal projections of wild-type adult mice. In Phase I, ~300 anatomically defined brain regions were chosen from the reference atlas ontology to comprehensively sample across major structures in the brain. Within each of the major parent structures, regions were selected as targets for anterograde tracing based on size, surgical

accessibility, and general scientific interest. Since the average viral infection area chosen for the initial study was approximately 400 – 700 µm in diameter, many small nuclei were not selected for Phase I. For larger regions, and regions with diverse topography, multiple injection sites were allocated. Based on the data resulting from Phase I and availability of appropriate Cre driver mice (see "Cre Mice Selection" below), more refined neuronal populations were targeted in the later phase of the project.

CRE MICE SELECTION

In Phase II, axonal projections from genetically-defined populations of neurons were studied. Mice engineered to express Cre recombinase under the control of unique gene promoters were injected with Cre-dependent rAAV vectors to fluorescently label Cre-expressing neurons in specific brain regions. The goals of Phase II were three-fold: (1) map axonal projections from discrete and/or otherwise difficult to access subcortical target areas using anatomically-restricted Cre expression, (2) map projections from specific functional cell types within subcortical brain areas and (3) map axonal projections from neurons in each cortical layer from 40 anatomically-defined cortical areas using mice with laminar-specific Cre expression. Specific Cre lines were identified that were best suited to meet these goals. Information on existing Cre mice was collected and curated from several sources, including GENSAT (http://www.gensat.org/cre.jsp), MGI creportal (http://www.creportal.org/), Cre-X-Mice (http://nagy.mshri.on.ca/cre_new/index.php), MMRRC (http://www.mmrrc.org/catalog/StrainCatalogSearchForm.jsp), NIH Neuroscience Blueprint Cre Driver Network (http://www.credrivermice.org/), our own Allen Institute Cre lines (http://connectivity.brain-map.org/transgenic), as well as literature searches for other Cre lines that may not be available yet in the above public repositories. Only Cre mice with transgene expression in the central nervous system, with documented or suspected neuronal expression of Cre, and with biologically interesting or anatomically restricted expression patterns were selected as candidates for further characterization. With these sources and criteria, approximately 340 Cre lines were identified with over 200 unique gene promoters. One hundred of these lines were further characterized in the Transgenic Characterization pipeline, which will become an important resource for choosing the most suitable Cre lines and target regions in each line for further study of axonal projections.

SERIAL TWO-PHOTON TOMOGRAPHY PIPELINE

Axonal projections labeled by rAAV tracers will be systematically visualized through the serial two-photon tomography pipeline. By coupling high-speed multi-photon microscopy with automated vibratome sectioning on a dimensionally stable tissue block, this imaging platform greatly reduces deformations commonly occurring with conventional section-based histological methods and provides a series of high-resolution images in pre-aligned 3-D space. This approach, thus, offers a strong foundation for the development of 3-D tools and connectivity models.

Mice

For Phase I, adult male C57BL/6J mice (stock 00064) were purchased from The Jackson Laboratory and acclimated to our facility for at least 5 days prior to surgery. For Phase II, Cre driver mice were obtained from various sources as described above as well as in the *Resources* white paper under the Documentation tab. The Cre mice were backcrossed to C57BL/6J mice to minimize genetic variance. For the majority of the study, mice backcrossed more than 5 times were used. Mice are 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 Tracer

The rAAV vectors used for Phases I and II of this study were purchased from the Penn Vector Core (University of Pennsylvania, Philadelphia, PA). Briefly, for Phase I (anatomical targeting in wild-type mice) the vector 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). For Phase II (Cre driver lines), we used a Cre-

dependent rAAV incorporating the flip-excision (FLEX) switch to control expression of EGFP from the CAG promoter (rAAV2/1.pCAG.FLEX.EGFP.WPRE.bGH). We found that rAAV serotype 1 produced the most widespread tropism throughout diverse brain areas, and therefore was chosen for this study. Methods used for the packaging, purification and determination of titer of 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). For the majority of target sites, 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 several of the most caudal medullary nuclei (*e.g.*, gracile nucleus and spinal nucleus of the trigeminal, caudal part), the calamus (at the floor of the 4th ventricle) is used as a registration point instead of Bregma. For many cortical areas, injections were made at two depths to label neurons throughout all six cortical layers and/or at an angle to infect neurons along the same cortical column. For laterally located cortical areas (e.g. orbital area, medial part; prelimbic area; agranular insular area), the injection was made at two adjacent ML coordinates for the same reason since the pipette angle required for injection along the cortical column is nearly 90° and was beyond our technical limit. The stereotaxic coordinates used for generating data available in the Atlas are listed in the *Injection Sites & Stereotaxic Coordinates* white paper under the Documentation tab.

Stereotaxic Injections of rAAV 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. For all injections utilizing Bregma as a registration point, an incision was made to expose the skull and Bregma and Lambda 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. For targeting caudal nuclei in the medulla, ketamine-anesthetized mice were placed in the stereotaxic frame with the nose pointed downward at a 45-60 degree angle. An incision was made in the skin at the base of the skull and muscles were bluntly dissected to reveal the posterior atlantooccipital membrane overlying the surface of the medulla. A needle was used to puncture the membrane, and the calamus was visualized. All mice received one unilateral injection into a single target region. Glass pipettes (inner tip diameter of 10-20 µm) loaded with virus were lowered to the desired depth from the pial surface of the brain. Currents were applied for iontophoresis of rAAV particles. The majority of injections were done using 3 µA at 7 sec on/7 sec off cycle for 5 min total. These settings resulted in infection areas of approximately 400 – 1000 µm, depending on target region. Reducing the current strength to 1 µA decreased the area of infected neurons, and was used when 3 µA currents produced infection areas larger than ~700 µm. Mice were quickly recovered after surgery and survived for 21 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. Brains were then rinsed briefly with PBS and stored in PBS with 0.1% sodium azide before proceeding to the next step. Agarose was used to embed the brain in a semisolid matrix for serial imaging. After removing residual moisture on the surface with a Kimwipe, the brain was placed in a 4.5% oxidized agarose solution made by stirring 10 mM $NaIO_4$ in agarose, then transferring through phosphate buffer and embedding in a grid-lined embedding mold to standardize placement of the brain in an aligned coordinate space (**Figure 2**). The agarose block was then left at room temperature for 20 minutes to allow agarose to solidify, and then covalent interaction between the brain tissue and the agarose was promoted by placing the block in 0.5% sodium borohydride in 0.5M sodium borate buffer (pH 9.0) overnight at 4°C. The agarose block was then mounted on a 1x3 glass slide using Loctite 404 glue and prepared immediately for serial imaging.

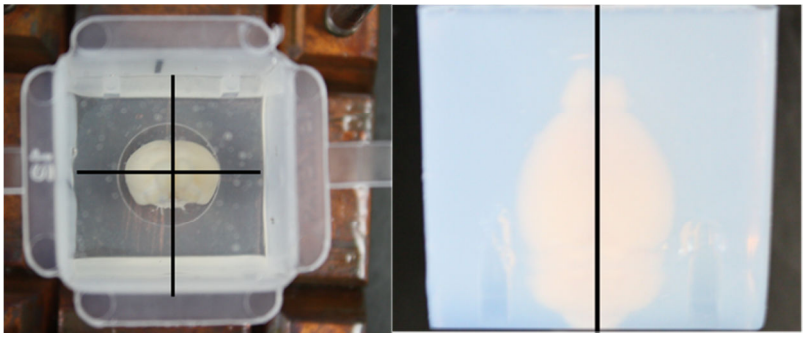

Figure 2. Embedding in agarose.
A brain was aligned in the middle of the agarose block so that the anterior-posterior axis is perpendicular to the bottom and parallel to the vertical axis of the mold. Top (left panel) and side (right panel) views are shown above.

Serial Two-Photon Tomography

Serial two-photon (STP) tomography is a recent technique that was developed at the Massachusetts Institute of Technology (Ragan, 2004, 2007) and commercialized by the support of several National Institute of Health SBIR grants to TissueVision, Inc. STP tomography's first application in the field of neuroscience was demonstrated at Cold Spring Harbor Labs (Ragan, 2012). Multi-photon image acquisition for the Atlas was accomplished using the TissueCyte 1000 system (TissueVision, Cambridge, MA) coupled with a Mai Tai HP DeepSee laser (Spectra Physics, Santa Clara, CA).

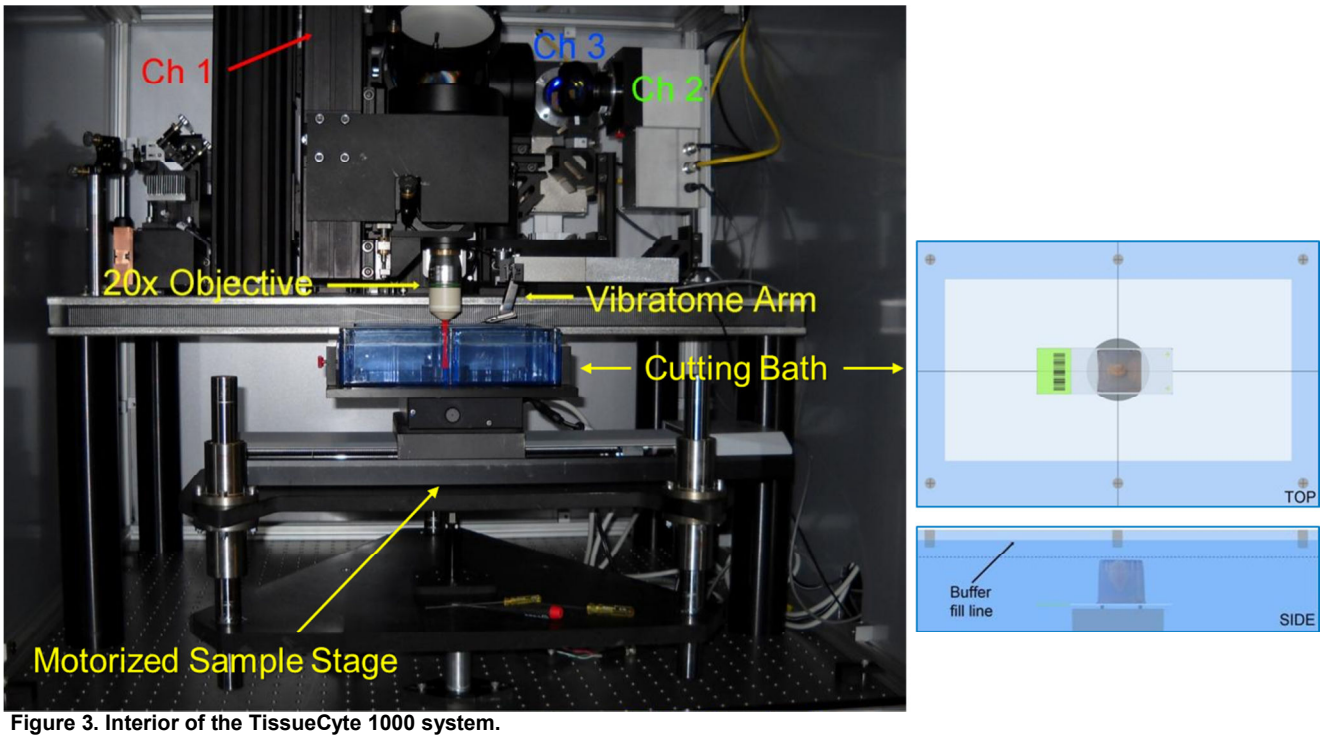

Figure 3. Interior of the TissueCyte 1000 system.
The embedded brain is placed in the middle of the cutting bath so that the leading edge of the agarose block is parallel to the blade (right panel). The cutting bath was filled with PBS-azide buffer and returned to the TissueCyte 1000 system. The motorized sample stage accurately moves the tissue block back and forth between objective lens and vibratome (left panel) to complete imaging of the whole mouse brain.

First, the mounted specimen was placed on the metal plate in the center of the cutting bath as illustrated in **Figure 3**. The cutting bath was filled with PBS with 0.1% sodium azide and placed onto the sample stage. To

achieve best results, a new vibratome blade was used for each specimen and aligned to be parallel to the leading edge of the specimen block.

Next, the top surface of the specimen block was brought up to the level of the vibratome blade by adjusting the sample stage height. The z-stage was set to slice at 100 µm intervals. Specimens were oriented for image acquisition to occur from the caudal to the rostral end. Boundaries for each edge of the specimen were defined, such that the XY scan area of the entire brain was estimated as shown in **Figure 4**. The specimen was illuminated with 925 nm wavelength laser. The excitation beam was directed to 75 µM below the cutting plane of each specimen by moving the objective using a piezo-controller (Physik Instrumente, Karlsruhe, Germany). This depth was selected as it is deep enough to minimize deformations at the cutting surface caused by vibratome sectioning but shallow enough to retain sufficient photon penetration for high contrast images. Thus, the optical plane of section is at 75 µM below the slicing surface, with an illumination field area of approximately 3-4 µM ellipsoidal depth.

A Zeiss 20x water immersion objective (NA = 1) was used to focus the light on the sample and to direct fluorescence through the emission path of the system. A 560 nm dichroic (Chroma, Bellows Falls, VT) split the emission light, and a 500 nm dichroic (Chroma) further split the emission for a total of three channels. The 593/40 (Chroma), 520/35 (Semrock, Rochester, NY) and 447/60 emission filter (Chroma) were used for the Red, Green and Blue channels, respectively. The three photomultiplier tubes (R3896, Hamamatsu, Bridgewater, NJ) were used to detect the emission at each channel. In order to scan a full tissue section, individual tile images were acquired, and the entire stage (Physik Instrumente) was moved between each tile. After an entire section was imaged, the X and Y stages moved the specimen to the vibratome, which cut a 100 µm section and returned the specimen to the objective for imaging of the next section. The blade vibrated at 60 Hz and the stage moved toward blade at 0.5 mm/sec during cutting.

Images from 140 sections were collected to cover the full range of mouse brain. It takes about 18 hours at a resolution of approximately 0.35 µm/pixel. Upon completion of imaging, sections were retrieved from the cutting bath and stored in PBS with 0.1% sodium azide at 4°C.

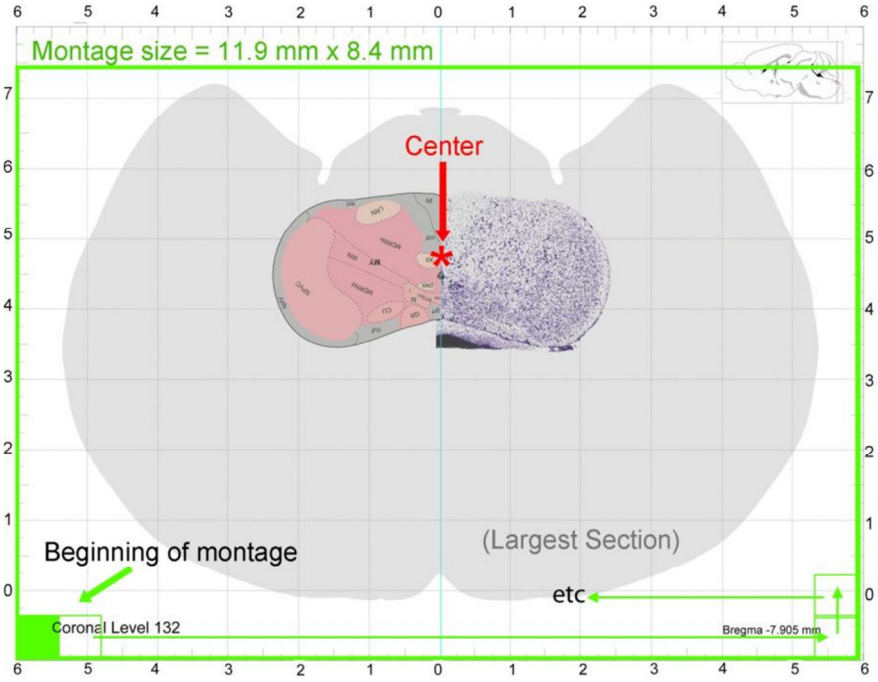

Figure 4. Scanning scheme of serial two-photon tomography.

The XY scan area consists of ~200 tiles (typically, 17 rows x 12 columns). Each tile is imaged at a resolution of 2080x2080 pixels. The stage moves the tissue block in a serpentine motion to put the next tile under the objective. Imaging of one section can be completed in ~8 min to achieve current resolution of 0.35 µm/pixel.

Data Processing

Once images were acquired, the Informatics Data Pipeline (IDP) managed image stitching, 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 project. The Informatics Data Processing whitepaper can be accessed at the Documentation tab on the Website.

Quality Control

Prior to public release of the data, quality control (QC) is implemented for all serial two-photon tomography data 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, edge cutoff, tessellation, and low signal strength may cause image series failure.

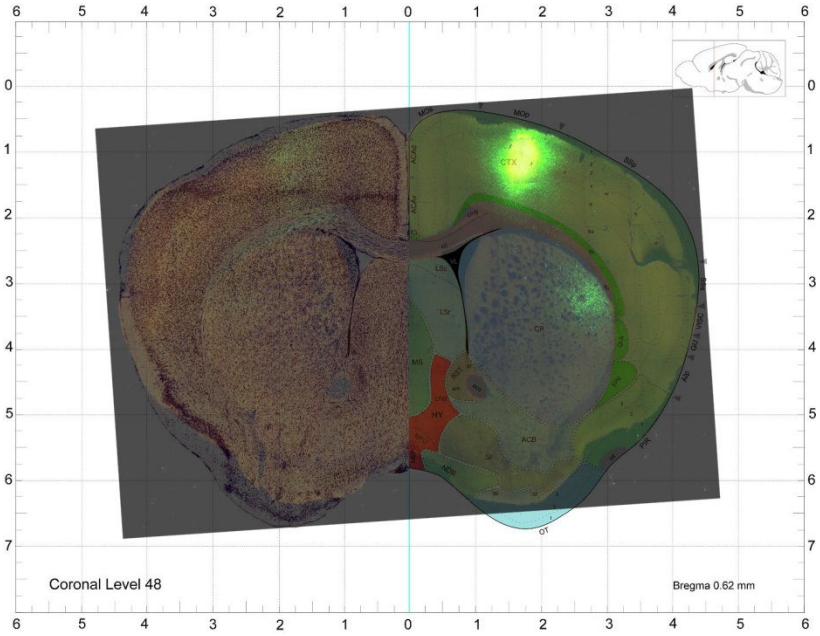

Figure 5. Example of injection site annotation.

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.

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). A representative section containing the injection site is selected and superimposed on the closest reference atlas plane, as exemplified in **Figure 5**. 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. Primary and secondary injection sites are also automatically populated by an informatics process that uses the manually drawn polygons. Our recently released Common Coordinate Framework (CCF) has introduced a disconnect between the manually identified injection structures based on

the previous version of Allen Mouse Brain Atlas, and the quantified injection summaries. As the creation of the CCF is an ongoing process, we have decided to keep both injection summaries on the results page. For instance, all manually annotated injection sites are listed under the “Manual Injection Summary” on the top left corner of the projection summary page whereas informatics-driven results are listed under the “Quantified Injection Summary” on the bottom left. Please see Documentation for further information on the CCF.

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