ALLEN INSTITUTE for BRAIN SCIENCE
Fueling Discovery

ALLEN **Mouse Brain Connectivity Atlas**

TECHNICAL WHITE PAPER: RETINAL PROJECTOME

OVERVIEW

The Allen Mouse Brain Connectivity Atlas – Retinal Projectome is designed as a three-dimensional, high resolution map of axonal projections from retinal ganglion cells (RGC) to the brain. Axonal projections originating from genetically-defined populations of RGCs are mapped on a standardized platform to generate a comprehensive database of neural projections. The same genetic tracing approach applied to the Allen Mouse Brain Connectivity Atlas is also utilized here. Cre driver mice with expression in RGC populations are combined with Cre-dependent viral mediated expression of fluorescent proteins to selectively label central axons from Cre-expressing cells. This project is a collaboration with Joshua Sanes, Ph.D., at the Center for Brain Science, Harvard University.

The Atlas consists of high resolution 2-D image data from the whole brain that can be viewed along with images of the corresponding whole mounted retina and associated reference datasets for each Cre line. Tools that enable 3-D visualization and spatial/ontological search of connectivity models through a combination of manual and informatics analyses are also available. The Retinal Projectome Atlas is expected to include:

Datasets

- Projection Mapping: Axonal projections mapped from approximately 30 Cre driver lines with expression in RGC populations, labeled by viral tracers and visualized using serial two-photon tomography.
- Transgenic Characterization: RGC transgene expression characterized in adults from approximately 30 Cre lines by Cre-dependent viral labeling and immunohistochemistry.

Key features

- 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 and corresponding retinal whole mount from 26 Cre driver lines.
- Transgenic Characterization data for each Cre driver line.

METHODOLOGY

CRE LINE SELECTION

Approximately 30 Cre driver lines were selected based on previously published work, or through screening of 100 Cre driver lines previously characterized for brain expression patterns at the Allen Institute for Brain Science (http://connectivity.brain-map.org/transgenic/). Lines with Cre expression in RGC populations, either multiple or single types, were chosen for central projection mapping in close collaboration with the laboratory of Joshua Sanes, Ph.D., at Harvard University. The specific morphological and/or functional types of RGCs labeled in each Cre line are not yet known for all these lines. One Cre line (Slc17a6-Cre) is a pan-RGC marker and is included in order to map the collective projections from all types of RGCs to the retinorecipient regions of the mouse brain. No wild-type control is used. Cre lines with data currently available are shown in **Table 1**.

Mice

Cre driver mice were obtained from various sources as described in the *Resources* white paper under the Documentation tab. Most 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 were group-housed (5 per cage) in micro ventilated cages with a 12 hour light/dark cycle. Purina Lab diet 5001 mouse food and water were given *ad libitum*. Surgeries were done in adult mice with an average age of postnatal day (P)56 ± 7 days.

Anterograde Tracer

Similar to Phase II of the Allen Mouse Brain Connectivity Atlas project, a Cre-dependent rAAV was used that incorporated the flip-excision (FLEX) switch to control expression of EGFP from the CAG promoter. rAAV2/2.pCAG.FLEX.EGFP.WPRE.bGH was purchased from the Penn Vector Core (University of Pennsylvania, Philadelphia, PA). The rAAV serotype 2 produced the most widespread infection across the retina without toxicity, and therefore was chosen for this study over serotype 1. 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.

Eye Injections of rAAV

Animals were anesthetized with ketamine/xylazine (100mg/kg ketamine, 10mg/kg xylazine). One drop of 0.5% proparacaine hydrochloride ophthalmic solution was applied as a topical anesthetic to the left eye. A small incision was made by inserting a sharp 30 gauge needle to the depth of the bevel just below the limbus of the eye. A blunt 33 gauge needle attached to a Hamilton syringe was inserted to withdraw 2µL of vitreous fluid. A second Hamilton syringe was used to inject 2µL of virus (rAAV2/2.pCAG.FLEX.EGFP.WPRE.bGH). At each of these steps, the needle was carefully inserted between the lens and the retina in order to not damage either. Petroleum ophthalmic ointment was then applied to the eye, and 8mg/kg of ketoprofen was administered as an analgesic.

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. rAAV injected eyes were dissected and a cautery was used to mark the dorsal orientation on each eye, which was then post-fixed in 4% PFA for 30-60 min. Brains and eyes were then rinsed briefly with PBS and stored in PBS with 0.1% sodium azide before proceeding to the next steps. Agarose was used to embed the brain in a semisolid matrix for serial imaging. After removing residual moisture on the surface of the tissue, the brain was placed in a 4.5% oxidized agarose solution with 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. 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 for serial imaging. Each retina was dissected entirely from the eye cup and marked to correspond with the location of cauterization.

Immunohistochemistry

All retinas were first briefly screened for native GFP fluorescence to ensure signal was present and there was good infection coverage across the extent of the retina. Then, retinas were processed as either whole mounts or for sectioning. For those retinas that were prepared as flattened whole mounts, they were first immunostained for GFP. Briefly, the retinas were blocked in 0.5% Triton X and 3% normal donkey serum in 1X PBS +0.1% azide on a shaker overnight at 4°C. The next day they were placed into primary antibody (chicken anti-GFP, Abcam, 1:1000) and incubated for 1 week on a shaker at 4°C. Then, they were washed in PBS and incubated with the secondary antibody (donkey anti-chicken Alexa Fluor 488, Jackson Immunoresearch, 1:500) on a shaker overnight at 4°C. The following day they were washed in PBS, relaxing cuts were made and the retina was transferred to filter paper and mounted and coverslipped on glass slides using Fluoromount-G with DAPI (Southern Biotech).

For characterization of morphology and co-localization with markers of RGC types, retinas were first sectioned onto glass slides at 20 µm using a cryostat, and stored at -20°C until use. For immunostaining, slides were thawed for 1 hour at room temperature. A border was drawn around the tissue with a hydrophobic pen, then the sections were rehydrated in PBS for 10 minutes, before being placed in blocking solution (3% normal donkey serum and 0.3% Triton X in PBS) for 45 minutes to 2 hours. Next, primary antibodies were diluted in blocking solution. Each slide was stained with one of four primary antibodies (guinea pig anti-VAChT, Chemicon 1:500; rabbit anti-CART, Phoenix Pharmaceuticals, 1:2000; goat anti-osteopontin, Santa Cruz, 1:1000; or mouse IgG1 anti-Brn3a, Millipore, 1:500) plus anti-GFP antibody (chicken anti-GFP, Abcam, 1:1000). Slides were incubated overnight in primary antibodies at 4°C. Then, slides were rinsed in PBS and incubated in secondary antibodies diluted in blocking solution for 2 hours at room temperature (donkey anti-chicken Alexa Fluor 488 for GFP, donkey anti-guinea pig, rabbit or mouse Alexa Fluor 555 for the other primary detection). After a final rinse in PBS, the slides were coverslipped using Fluoromount-G with DAPI.

Imaging of Retina

After dissection, retinas that have been dissected, flat-mounted to slides and histologically stained were imaged via semi-automated protocols by multichannel fluorescence microscopy (Olympus VS series; Olympus, Center Valley, PA). A 10X objective with autofocus was used to acquire full-color images with a resolution of approximately 0.64µm per pixel. The images taken from the flat-mounted retinas are shown with the orientation of dorsal side up, ventral side down, temporal to the left, and nasal to the right.

Serial Two-Photon Tomography

Serial two-photon (STP) tomography was done for all the brains as described in the Overview document located under the Documentation tab.

Table 1. Cre driver lines used for RGC projection mapping.

Cre Line	Alias	Originating Lab (Donating Investigator)	Public Repository	Public Repository Stock #	Official Strain Name
Calb2-IRES-Cre	Cr-IRES-Cre	Z. Josh Huang	JAX	010774	B6(Cg)-*Calb2*$^{tm1(cre)Zjh}$/J
Cart-Tg1-Cre		Allen Institute for Brain Science	JAX	009615	STOCK Tg(Cartpt-cre)1Aibs/J

Cdh4-CreER	KO4CAD-Cre	Joshua Sanes			
Cdh6-CreER	KO6CAD-Cre	Joshua Sanes			
Cnnm2-Cre_KD18		Nathaniel Heintz and Charles Gerfen	MMRRC	030951	STOCK Tg(Cnnm2-cre)KD18Gsat/Mmucd
Crh-IRES-Cre (BL)		Brad Lowell			
Cux2-IRES-Cre		Ulrich Mueller	MMRRC	031778	B6(Cg)-Cux2<tm1.1(cre)Mull>/Mmmh
Drd1a-Cre	D1-Cre	Richard Palmiter			
Etv1-CreERT2	ER81-CreERT2	Z. Josh Huang	JAX	013048	B6(Cg)-*Etv1*$^{tm1.1(cre/ERT2)Zjh}$/J
Foxp2-IRES-Cre		Richard Palmiter			
Gal-Cre_KI87		Nathaniel Heintz and Charles Gerfen	MMRRC	031060	STOCK Tg(Gal-cre)KI87Gsat/Mmcd
Gpr26-Cre_KO250		Nathaniel Heintz and Charles Gerfen	MMRRC	033032	STOCK Tg(Gpr26-cre)KO250Gsat/Mmucd
Grik4-Cre	G32-4	Susumu Tonegawa	JAX	006474	C57BL/6-Tg(Grik4-cre)G32-4Stl/J
Htr2a-Cre_KM207		Nathaniel Heintz and Charles Gerfen	MMRRC	031150	STOCK Tg(Htr2a-cre)KM207Gsat/Mmucd
JAM2-Cre		Joshua Sanes			
JAM2-CreER		Joshua Sanes			
Kcng4-Cre	KOKCNG-Cre	Joshua Sanes			
Neto1-Cre		Joshua Sanes			
Pcdh9-Cre_NP276		Nathaniel Heintz and Charles Gerfen	MMRRC	036084	STOCK Tg(Pcdh9-cre)NP276Gsat/Mmcd
Pvalb-IRES-Cre		Silvia Arber	JAX	008069	B6;129P2-*Pvalb*$^{tm1(cre)Arbr}$/J
Satb2-Cre_MO23		Nathaniel Heintz and Charles Gerfen	MMRRC	32908	STOCK Tg(Satb2-cre)MO23Gsat/Mmucd
Scnn1a-Tg3-Cre		Allen Institute for Brain Science	JAX	009613	B6;C3-Tg(Scnn1a-cre)3Aibs/J

Sdk2-CreER	KO2SDK-Cre	Joshua Sanes			
Slc17a6-IRES-Cre	VGLUT2-ires-Cre	Brad Lowell			
Slc18a2-Cre_OZ14		Nathaniel Heintz and Charles Gerfen	MMRRC	34814	STOCK Tg(Slc18a2-cre)OZ14Gsat/Mmcd
Thy1-Cre		Fred Van Leuven	JAX	006143	FVB/N-Tg(Thy1-cre)1Vln/J