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Laser-scanning photostimulation of optogenetically targeted forebrain circuits

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Corresponding Author:	Charles C. Lee, Ph.D. Louisiana State University Baton Rouge, LA UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Louisiana State University
Corresponding Author's Secondary Institution:	
First Author:	Charles C. Lee, Ph.D.
First Author Secondary Information:	
Order of Authors Secondary Information:	
Abstract:	<p>The sensory forebrain is composed of intricately connected cell types, of which functional properties have yet to be fully elucidated. Understanding the interactions of these forebrain circuits has been aided recently by the development of optogenetic methods for light-mediated modulation of neuronal activity. Here, we describe a method for examining the functional organization of forebrain circuits in vitro using laser-scanning photostimulation of channelrhodopsin, expressed optogenetically via viral-mediated transfection. This approach also exploits the utility of cre-lox recombination in transgenic mice to target expression in specific neuronal cell types. Following transfection, neurons are physiologically recorded in slice preparations using whole-cell patch clamp to measure their evoked responses to laser-scanning photostimulation of channelrhodopsin expressing fibers. This approach enables an assessment of functional topography and synaptic properties. Morphological correlates can be obtained by imaging the neuroanatomical expression of channelrhodopsin expressing fibers using confocal microscopy of the live slice or post-fixed tissue. These methods enable functional investigations of forebrain circuits that expand upon more conventional approaches.</p>
Corresponding Author E-Mail:	cclee@lsu.edu
Additional Information:	
Question	Response
Order of Authors:	Charles C. Lee, Ph.D. Ying-Wan Lam, Ph.D. Kazuo Imaizumi S. Murray Sherman
Author Comments:	March 3, 2013 Mr. Matthew Kramer: Thank you again for selecting our submission for the Leica Publication Grant Award. We now submit our invited article entitled "Laser-scanning photostimulation of optogenetically targeted forebrain circuits," which we believe will appeal to a broad scientific audience. Our protocol combines novel optogenetic techniques with laser-scanning photostimulation to assess features of functional topography in brain slice preparations.

Please find attached our submission: the manuscript text with figures.
Thank you again for inviting this submission and awarding us with a Leica Publication Grant for publication of this article in the Journal of Visualized Experiments. We look forward to your response.

Best regards,

Charles C. Lee, Ph.D.
Assistant Professor
Department of Comparative Biomedical Sciences
LSU School of Veterinary Medicine
Skip Bertman Drive
Baton Rouge, LA 70803

In revision:

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Laser-scanning photostimulation of optogenetically targeted forebrain circuits

Charles C. Lee¹, Ying-Wan Lam², Kazuo Imaizumi¹, S. Murray Sherman²

¹Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University, Baton Rouge, LA 70803

²Department of Neurobiology, University of Chicago, Chicago, IL 60637

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Correspondence to: Charles C. Lee
Department of Comparative Biomedical Sciences
Louisiana State University
School of Veterinary Medicine
Skip Bertman Drive
Baton Rouge, LA 70803

Email: cclee@lsu.edu
Telephone: (225) 578-9032
Fax: (225) 578-9895

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SHORT ABSTRACT

We describe a method for characterizing the functional topography and synaptic properties of forebrain circuits using an optogenetic approach to photostimulate neuronal populations *in vitro*.

LONG ABSTRACT

The sensory forebrain is composed of intricately connected cell types, of which functional properties have yet to be fully elucidated. Understanding the interactions of these forebrain circuits has been aided recently by the development of optogenetic methods for light-mediated modulation of neuronal activity. Here, we describe a protocol for examining the functional organization of forebrain circuits *in vitro* using laser-scanning photostimulation of channelrhodopsin, expressed optogenetically via viral-mediated transfection. This approach also exploits the utility of cre-lox recombination in transgenic mice to target expression in specific neuronal cell types. Following transfection, neurons are physiologically recorded in slice preparations using whole-cell patch clamp to measure their evoked responses to laser-scanning photostimulation of channelrhodopsin expressing fibers. This approach enables an assessment of functional topography and synaptic properties. Morphological correlates can be obtained by imaging the neuroanatomical expression of channelrhodopsin expressing fibers using confocal microscopy of the live slice or post-fixed tissue. These methods enable functional investigations of forebrain circuits that expand upon more conventional approaches.

INTRODUCTION

The elaborate circuitry of the mammalian forebrain poses an experimental challenge to dissecting the functional roles of its intertwined components. Untangling these relationships has been aided recently by the development of optogenetic methods for specifically exciting and inhibiting neuronal populations and their projections¹⁻³. These approaches make use of the genetically controlled expression of membrane channels responsive to light⁴⁻⁶. Of particular note, channelrhodopsin, a light-gated ion channel, depolarizes neuronal membranes following optical stimulation, leading to excitation of the expressing neuron and its projections^{4,7}. Conversely, halorhodopsin, a light-gated chloride pump, hyperpolarizes the membrane, thus inhibiting the targeted projection^{6,8,9}. Several variants of these opsins have been engineered, multiple vectors produced for transfection and expression in neuronal populations of interest, and transgenic mouse lines engineered that constitutively express these opsins or that enable cre-lox mediated control of their expression^{1,2,10,11}.

The utility of these optogenetic methods and materials has revolutionized modern experimental neuroscience, enabling the dissection of the role of specific neural circuits in the physiology and behavior of the organism¹²⁻¹⁵. In particular, these methods can be powerfully applied to the functional dissection of brain circuits using *in vitro* slice preparations¹⁶⁻²⁰. In this context, one of the main advantages of this method is the ability to characterize the physiology of long range projections that are difficult, if not impossible, to preserve in a thick slice, such as the commissural connections between cerebral cortical hemispheres¹⁶. Furthermore, this technology enables manipulations not possible with other methods, such as the selective inhibition of projections with fine temporal precision using halorhodopsin^{6,8,9}. Moreover, the cell-type specific expression of these opsins allows isolated components in a neural circuit to be selectively stimulated or inhibited^{1,2,11,15,21}. As such, these novel optogenetic tools enable the unprecedented analysis of the neuronal contributions to physiology and behavior.

Here, we describe a protocol for expressing channelrhodopsin in the mouse brain using adeno-associated viral (AAV) vectors^{1,2}. Specific transfection of targeted neuronal populations can be accomplished using this protocol, with the appropriate choice of promoter constructs or transgenic mice that express Cre-recombinase in the targeted neurons of interest and a 'floxed' viral construct^{1,11,15,21} (Fig. 1). As examples here, we examine the corticocortical projections between the primary visual cortex (V1) and the secondary visual cortex (V2) and we also

investigate the layer 6 corticothalamic projection from the primary somatosensory cortex (S1) to the ventroposterior medial nucleus (VPM). We combine this specific expression with laser-scanning photostimulation *in vitro* to assess synaptic physiology and functional topography. The setup presented here enables the precise spatial positioning and control of the loci of photostimulation. Finally, we describe the visualization of expression patterns using confocal microscopy.

PROTOCOL

1. Surgery and Injections

1.1. Anesthetize mice using an intraperitoneal injection of ketamine (100-120 mg/kg) and xylazine (5-10 mg/kg).

1.1.1. Assess sedation using strong toe pinches until no responses are elicited. For all animal procedures described, modify to follow local IACUC guidelines as necessary.

1.2. Place sedated mice in a stereotaxic device by securing the head and ears in their respective holders.

1.3. Shave the scalp with a razor and sterilize the area with betadine swabs.

1.4. Using a scalpel, cut a midline incision and reflect the skin and underlying muscles above the area to be injected.

1.5. Rotate a scalpel blade as a trephine to perform a craniotomy above the region to be exposed. Alternatively, use a small drill bit attached to a Dremel to perform the craniotomy.

1.5.1. To perform the craniotomy, place scalpel blade or drill bit on surface of skull and slowly rotate until enough bone has been excavated to reveal the pia mater underneath.

1.5.2. Once the initial excavation is made, enlarge the craniotomy using fine forceps to carefully remove bone from the edges.

1.6. Fill a 1 μ l syringe with the viral solution to be injected (UNC Vector Core).

1.6.1. To enhance transfection efficacy, prepare viral solution with 4 μ g/ml polybrene.

1.7. Lower the needle into the area to be injected and pressure infuse 200 nl of solution containing virus with a syringe pump.

1.7.1. Let syringe sit for 10 minutes before withdrawing the needle.

1.8. Suture the skin with non-absorbable monofilament nylon and cover the incision with antibiotic ointment.

1.9. Inject buprenorphine (0.05-0.1mg/kg) for post-operative analgesia.

1.10. Allow animals to post-operatively recover for 14-21 days to allow for adequate expression of channelrhodopsin.

2. Slice Preparation

2.1. Following the recovery period, deeply anaesthetize the animal by placing it in an enclosed chamber containing a small, gauze-filled receptacle, such as a 10 ml beaker, with a few drops of isoflurane.

2.2. Dissect the brain by decapitation followed by midline and frontal cuts to the skull.

2.3. Peel apart the skull from the midline to expose the brain.

2.4. Carefully remove the brain using a fine scoop spatula to separate the cranial nerves from the base of the brain.

2.5. Quickly place dissected brain in small 100 ml beaker containing chilled, ice-cold artificial cerebrospinal fluid (ACSF; in mM: 125 NaCl, 3 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂, 25 NaHCO₃, 25 glucose).

2.6. Remove brain from ACSF and place on a microscope slide covered with filter paper pre-wetted with ACSF.

2.7. Make a blocking cut of the brain in the desired orientation using a clean razor blade.

2.8. Affix the blocked surface of the brain to cutting stage using instant adhesive glue.

2.9. Transfer stage to a vibratome cutting chamber (kept in freezer until ready for cutting).

2.10. Pour ice cold ACSF into cutting chamber.

2.11. Slice brain sections at 400-500 μ m using a vibrating tissue slicer.

2.12. Transfer brain slices to a holding chamber containing oxygenated ACSF, and incubate at 30°C for at least 1 hour before experiment.

3. Physiological Recording

3.1. After the incubation period, transfer a slice to a recording chamber on a modified microscope stage.

3.2. Secure the slice in the bath during the experiment using a few threads of nylon filaments attached to a platinum wire slice holder.

3.3. Perfuse the slice with ACSF constantly.

3.4. Pull recording pipettes to a tip resistance of 3-6 M Ω when filled with intracellular solution containing the following (in mM): 127 K-gluconate, 3 KCl, 1 MgCl₂, 0.07 CaCl₂, 10 HEPES, 2 Na₂-ATP, 0.3 Na-GTP, 0.1 EGTA. The intracellular solution should have pH 7.3, adjusted with KOH or gluconic acid and the osmolality of 280-290 mOsm.

3.5. Using a standard visualized whole-cell recording setup, place recording pipette near neuron to be recorded while applying positive pressure^{22,23}.

3.6. Release positive pressure while applying suction to form gigaohm seal.

3.7. Apply brief suction to break membrane and form whole-cell configuration recording.

3.8. Record the responses of neurons in either voltage or current clamp.

4. Laser-scanning photostimulation

4.1. Start data acquisition software and hardware, described in previous reports²³⁻²⁹.

Note: We have utilized both Tidelwave²⁵ and, more recently, Ephus²⁶ acquisition programs, written in Matlab and developed by the laboratory of Karel Svoboda at Janelia Farms³⁰. For a full description of laser-scanning photostimulation in Ephus, see Suter et al. (2010) and the step-by-step guide³⁰.

Note: The setup described in this article is derived from that described by Shepherd (2012)²³. The laser beam is guided via an open tube system of mirrors to the top port of the microscope through the objective lens onto the slice (Fig. 2).

4.2. Divert a small portion of the laser onto a photodiode with a microscope coverslip placed in the laser path and use the current output from this photodiode to monitor the laser intensity during experiments.

4.2.1. Calibrate the photodiode current output recorded in the software to the measured laser power at the back focal plane of the objective using a power meter during equipment setup²³.

4.3. Control the laser power by rotating a variable neutral density wheel. The laser power used varied between 25 to 70 mW (at the back focal plane) in our experiments.

4.4. Set the length and timing of the laser stimulating by modifying the pulse parameters for the laser and shutter. Laser stimulation is typically 2 ms long.

4.5. Capture image of the region to be stimulated using the 'Grab Video' function.

4.6. Select the stimulation grid according to desired dimensions. We typically use a 16x16 stimulation array with 80 μ m spacing between adjacent rows and columns.

4.7. Overlay the mapping grid with the acquired image and adjust the x, y and degree offset to the desired location and orientation.

4.8. Activate the 'Map' function to start stimulation and acquisition of data.

4.9. Following acquisition of photostimulation evoked responses, carry out analysis of mapped data using either the off-line map analysis program in Tidelwave²⁵ or the 'MapAnalysis' program in Ephus²⁶.

5. Fixed Slice Imaging

5.1. After recordings, transfer brain slice to 4% paraformaldehyde (PFA) in 0.01 M PBS (pH7.4) overnight at 4 °C.

5.2. Transfer the slice in 30% sucrose in 4% PFA/0.01 M PBS overnight at 4 °C.

5.3. Cut 50 µm sections using a cryostat and collect in 0.01 M PBS.

5.4. Rinse sections in 0.01 M PBS twice at room temperature.

5.5. Mount sections and coverslip in an appropriate antifade medium.

5.6. Transfer sections to the confocal microscope.

5.7. Focus into the ROI using white light.

5.8. Choose the wavelength, imaging size, and repetition for image acquisition. For YFP, we use 514 nm (Ex)/527 nm (Em).

REPRESENTATIVE RESULTS

Laser-scanning photostimulation of projections between the primary visual area (V1) and the secondary visual area (V2) in the mouse was accomplished following injections of pAAV-CaMKIIa-hChR2(H134R)-EYFP into V1 of a Balb/C mouse (Figure 3). The CaMKIIa promoter in this viral construct drives specific expression of channelrhodopsin-EYFP in excitatory glutamatergic neurons^{1,15}. After preparation of slices for physiological recording, expression of channelrhodopsin-EYFP fibers, i.e. epifluorescence of the conjugated EYFP, was observed traversing from V1 to V2 (Figure 3A). In addition, the corticothalamic fibers in the lateral geniculate nucleus (LGN) were also observed (Figure 3A). Whole-cell patch clamp recordings from a neuron in V2 revealed EPSCs elicited following laser-scanning photostimulation of channelrhodopsin-EYFP expressing fibers in the lower layers of V2 (Figure 3B).

In another example, laser-scanning photostimulation of the corticothalamic projections from layer 6 of the primary somatosensory cortex (S1) to the ventroposterior nucleus (VP) was accomplished using this protocol. Here, we used a Cre-Lox approach to specifically target channelrhodopsin expression in the layer 6 neurons and corticothalamic projections of interest. Explicitly, we injected pAAV-Ef1a-DIO-hChR2(H134R)-EYFP-WPRE-pA into S1 of B6.Cg-Tg(Ntsr1-cre)GN220Gsat/Mmcd mice (Figures 1, 4). This transgenic mouse strain expresses

Cre-recombinase specifically in layer 6 corticothalamic neurons²¹, and therefore results in ChR-YFP expression only in those neurons following transfection by the ‘floxed’ AAV construct. After preparation of slices, channelrhodopsin-EYFP fibers were observed in the layer 6 neurons and fibers projecting to layer 4 and VP (Figure 4A,B). Physiological recording from a VPM neuron using whole-cell patch clamp revealed that EPSCs could be elicited following laser-scanning photostimulation near the recorded neuron (Figure 4C,D).

The morphology of labeled fibers was assessed more readily following physiological recordings. Here, we resectioned (50 μ m) slice preparations with channelrhodopsin-EYFP expression in layer 6 corticothalamic neurons (Figure 5). This revealed a fine pattern of labeled fibers traversing from layer 6 to layer 4, where they terminated and arborized extensively (Figure 5A). In addition, corticothalamic fibers were observed entering the white matter traversing towards the thalamus (Figure 5A,B).

TABLES AND FIGURES

Figure 1. Schematic illustration of targeted expression of channelrhodopsin via a Cre-Lox approach. Transgenic mice expressing Cre-recombinase in the neuronal populations of interest are injected with a ‘floxed’ virus encoding channelrhodopsin fused to a fluorescent protein, such as YFP. Following transfection, only neurons with Cre-recombinase will express channelrhodopsin-EYFP.

Figure 2. Schematic illustration of photostimulation setup. (A) Diagram of the laser-scanning photostimulation setup. The laser beam is guided successively through an open tube system to a shutter and a neutral density (N.D.) filter. A coverslip reflects part of the beam to a photodiode connected to an amplifier for laser power measurements. Mirror galvanometers are used to position the laser beam through a scan lens, which guides the beam through a side port on the top of the microscope. The beam is then reflected down through the objective lens to the slice preparation using a dichroic mirror. For further details see Lam and Sherman (2005) and Shepherd (2012). (B) Example of the photostimulation grid pattern for the representative experiment shown in Figure 3. The 16x16 stimulation grid is overlayed on a photomicrograph of the slice preparation. A red star indicates the location of a neuron recorded using whole-cell patch clamp. The laser-beam position is guided to each of the 256 positions in the stimulation grid by computerized positioning of the mirror galvanometers. The laser positions of the 1st 5 stimulation sites are depicted in the figure. The responses from the recorded neuron to photostimulation at each of the sites is depicted in Figure 3.

Figure 3. Laser-scanning photostimulation of corticocortical projections from primary visual cortex (V1) to secondary visual cortex (V2) in the mouse. (A) Epifluorescent image of the slice preparation showing channelrhodopsin-EYFP fibers from V1 to V2 and in the lateral geniculate nucleus (LGN). Specific expression of channelrhodopsin-EYFP in excitatory glutamatergic neurons is driven by a viral construct containing the CaMKIIa promoter. (B) Excitatory postsynaptic currents (EPSCs) elicited from a neuron in V2 following laser-scanning photostimulation. Red star indicates recording site. Scale bars: (A) 500 μ m, (B) 200 pA, 100 ms.

Figure 4. Laser-scanning photostimulation of corticothalamic projections from primary somatosensory cortex (S1) to the ventroposterior nucleus (VP) in the mouse. (A) Epifluorescent

image of the slice preparation showing channelrhodopsin-EYFP fibers in S1. (B) Epifluorescent image of labeled-fibers in VP. (C) Excitatory postsynaptic currents (EPSCs) elicited from a VP neuron following laser-scanning photostimulation. (D) Plot of mean EPSCs. Red star indicates recording site. Boxed traces in (C) correspond with boxed values in (D). Scale bars: (A) 250 μm , (B) 250 μm , (C) 100 pA, 100 ms.

Figure 5. Imaging of layer 6 neurons expressing channelrhodopsin-EYFP in the primary somatosensory cortex (S1) of the mouse. (A-B) Labeled neurons in layer 6 and fibers projecting to layer 4 and the white matter. Scale bars: (A) 250 μm , (B) 250 μm .

DISCUSSION

The described protocol details an approach for the *in vitro* assessment of functional topography and physiology of neuronal circuits, which is not limited to the investigation of forebrain circuits, i.e. those of the thalamus and cortex. Several viral constructs for optogenetic transfection have been made available to the scientific community and are readily obtainable from the UNC Vector Core. For a description of viral constructs readily available, see the UNC Vector Core³¹, the optogenetics resource page at the Deisseroth Lab at Stanford University³² and the Boyden synthetic neurobiology lab at MIT³³. Variants of these opsins have been developed by these labs and enable wide-ranging modes for controlling neuronal excitability^{1,2}. Obtaining these vectors from the UNC Vector Core requires a Material Transfer Agreement from the originating labs. In this protocol, we note that the addition of polybrene enhances transfection efficacy. In addition, another variable influencing expression is the post-surgical recovery period, which we note that a period of at least 14 days is sufficient to obtain robust expression in the forebrain pathways of mice.

For the targeted transfection of specific neuronal groups, several Cre-transgenic mouse lines have been developed, which can be used in combination with ‘floxed’ reversed open reading frame viral constructs that have been developed¹¹. Several strains pertinent to neuroscience research are available through the Mutant Mouse Regional Resource Centers³⁴. Atlases of Cre-recombinase expression patterns in these transgenic lines are offered through the GENSAT database³⁵. In addition, Jackson Labs offers several Cre-transgenic strains, as well as others that constitutively express opsins in specific neuronal groups. A critical step to utilizing these mouse lines is to factor in the time necessary for obtaining and establishing a breeding colony, which may vary based on the particular strain of interest.

For photostimulation, we have utilized and adapted the setup developed by the Shepherd Lab at Northwestern University, described in a recent protocol²⁹. Although optimized for glutamate uncaging, this setup is amenable also for photostimulation of channelrhodopsin without modification. This setup enables precise spatial control and positioning of the loci of photostimulation, which enables an assessment of the functional topography of channelrhodopsin innervation of recorded neurons. Although spatial control is enhanced with this method of photostimulation, it is more cost prohibitive than methods utilizing LED based field illumination²⁰. In addition, the non-uniformity of brain tissue is a limitation of optogenetics, or any techniques involving manipulation of light. In practice though, it is rarely a factor since results are interpreted mostly in a qualitative manner. In case more quantitative comparison between brain regions is needed, one can always use maximum light intensity to achieve "saturated" responses.

In addition, we have used both Tidelwave and Ephus software programs written in Matlab for controlling the laser-scanning photostimulation and data acquisition^{25,26}. However, it is recommended that those setting up a similar system employ the newer Ephus software²⁶, which has support available through the Janelia Farms development site³⁰. A key step is choosing which of these software packages to use, since their hardware requirements differ slightly. In addition, the map analysis programs are tailored to their respective data acquisition programs.

Several of these steps can be modified to suit the practitioner's particular setup. Injections of viral solutions can be performed with delivery mechanisms besides a Hamilton Syringe, e.g. capillary glass pipettes pulled to tip diameters of ~40 μm also work well. For surgeries, a non-stereotaxic head holder can be substituted if such coordinates are not required for guiding injections. In addition, ACSF and intracellular solution compositions may be altered depending on the particular experimental requirements, e.g. substituting a cesium or high chloride intracellular solution to isolate inhibitory currents. Also, an alternative to imaging of expression patterns in fixed slices may also be done in the live slice on a suitable imaging rig with a bath perfusion system.

In summary, this protocol will enable the practitioner to characterize the functional topography and synaptic physiology of projections *in vitro*, which may not be possible using standard methods, e.g. characterizing long-range projections that are unable to be preserved in a slice. The transfection protocol described can be applied to *in vivo* optogenetic experiments^{1-3,12,14}, since the delivery mechanism is the same. Finally, although this protocol describes transfection of mice, many of the viral vectors available can be used to theoretically transfect and express these opsins in a wide range of mammalian species, thus enabling a myriad of comparative and species-specific studies¹⁻³.

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DISCLOSURES

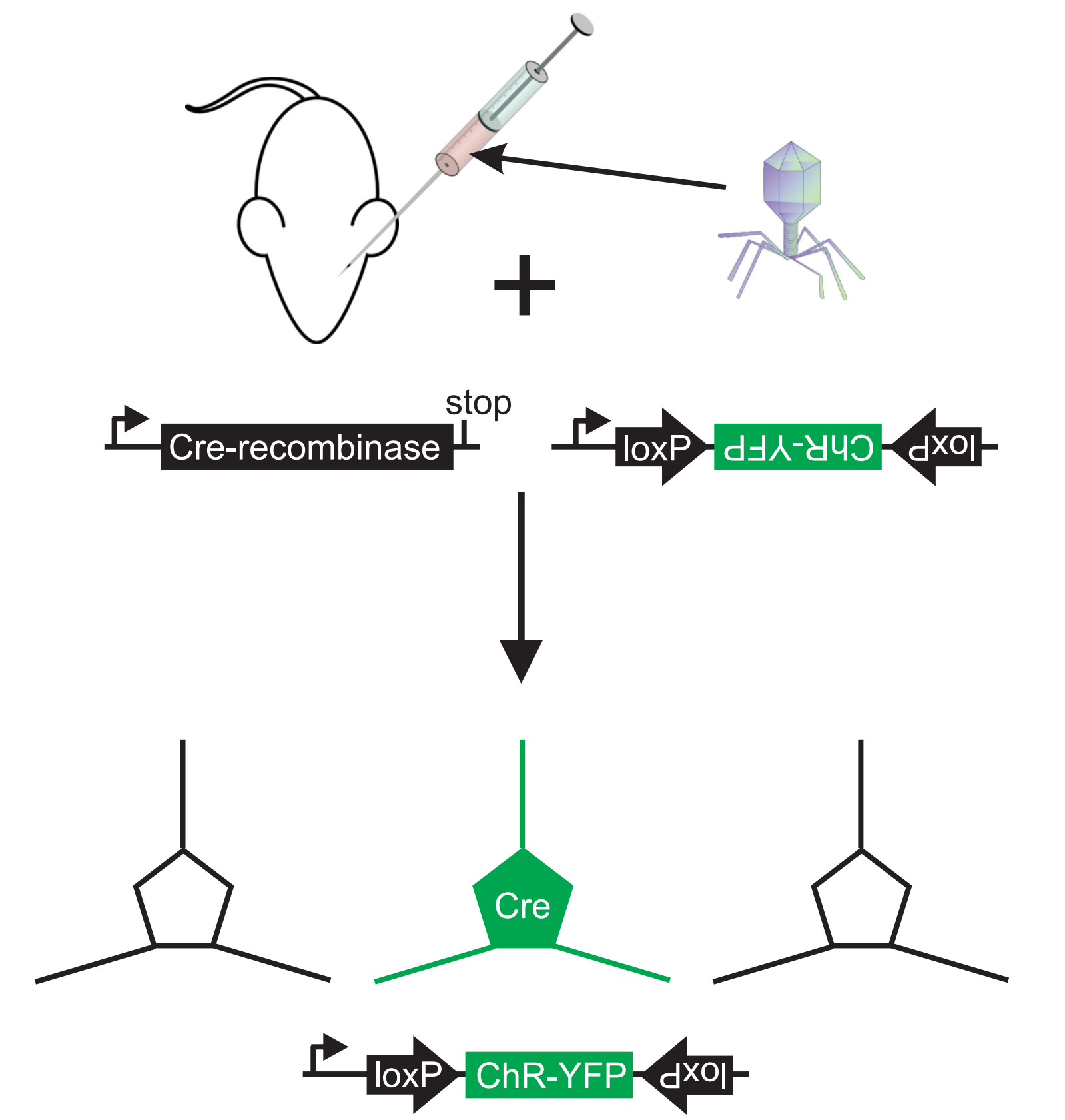
The authors declare they have no competing interests.

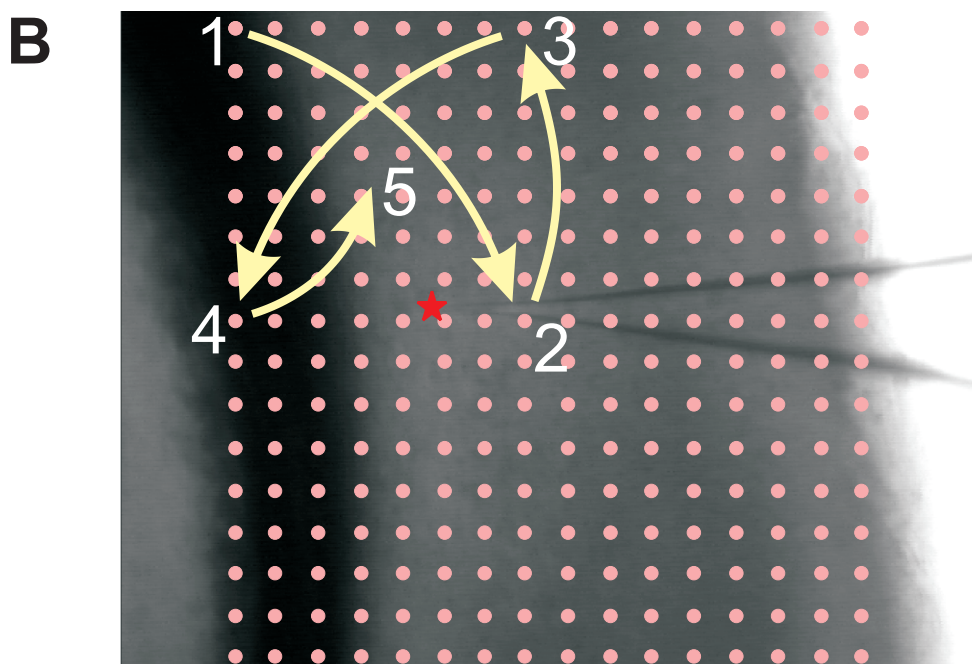
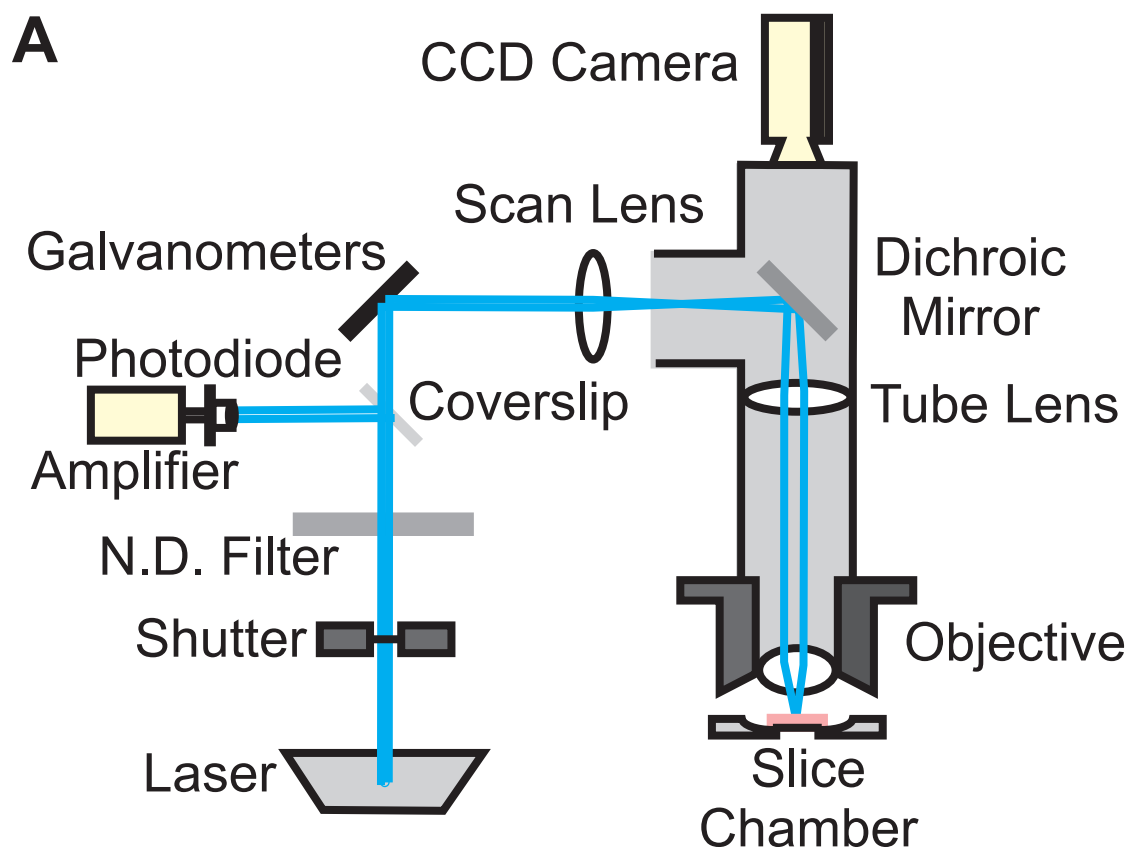
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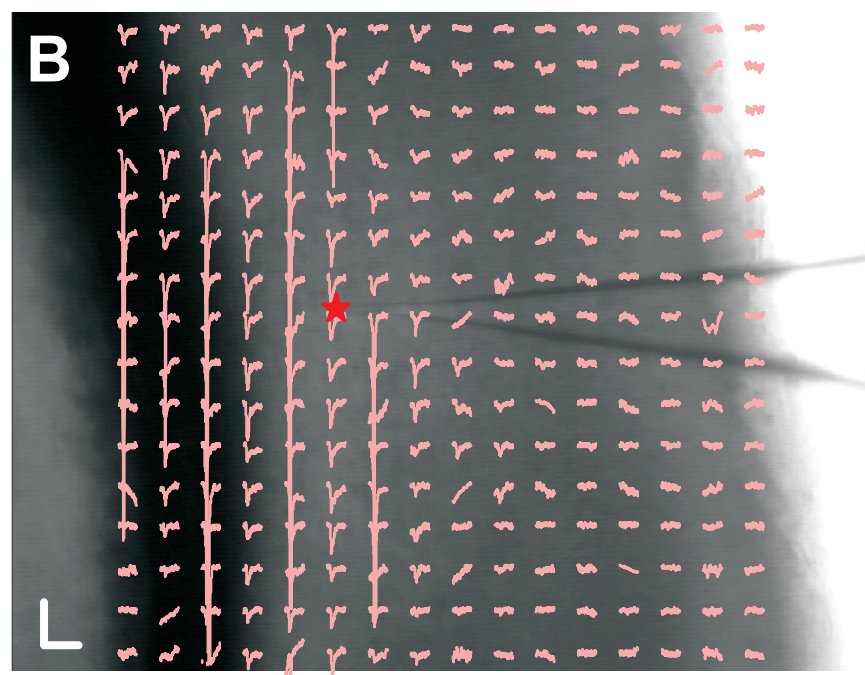
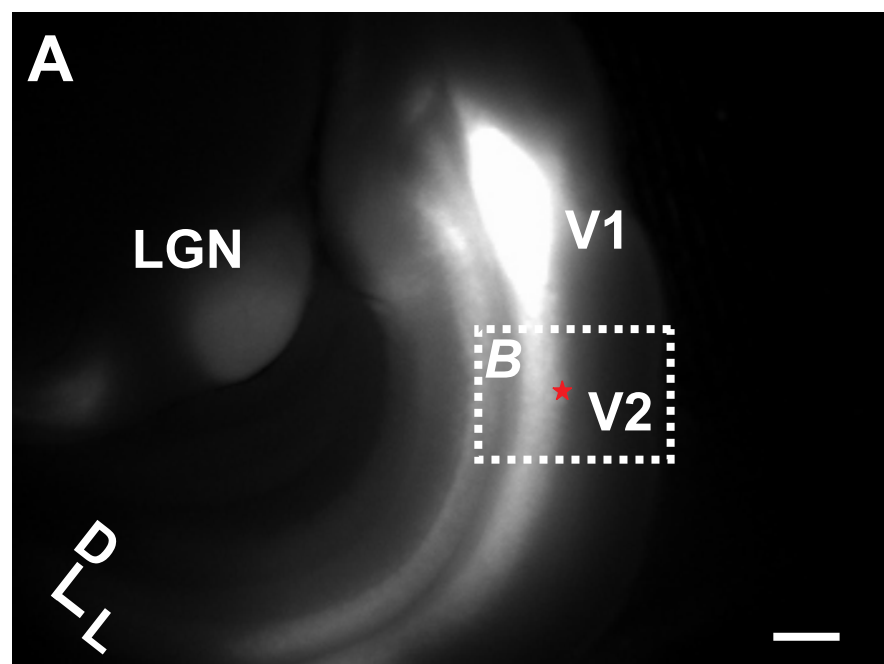
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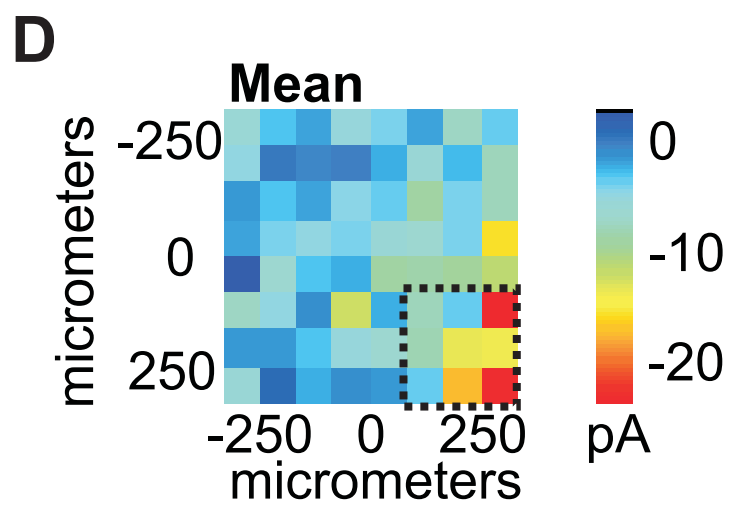
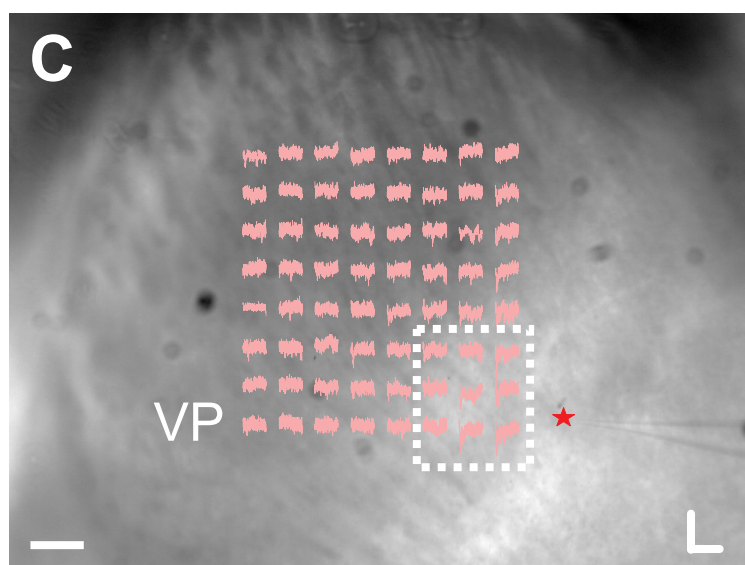
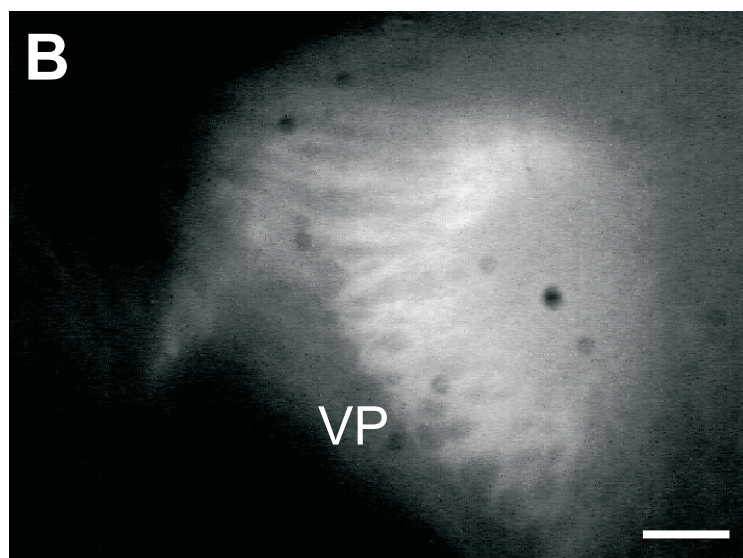
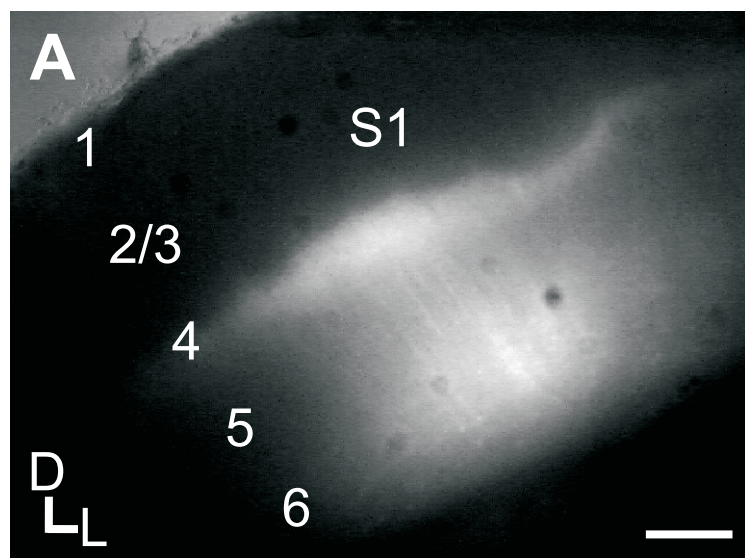
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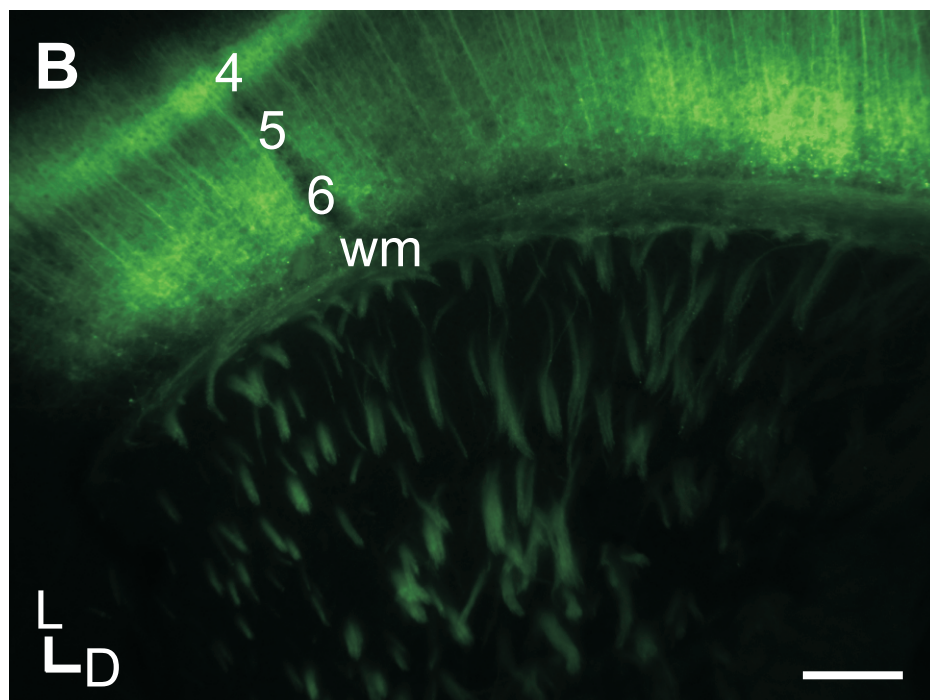
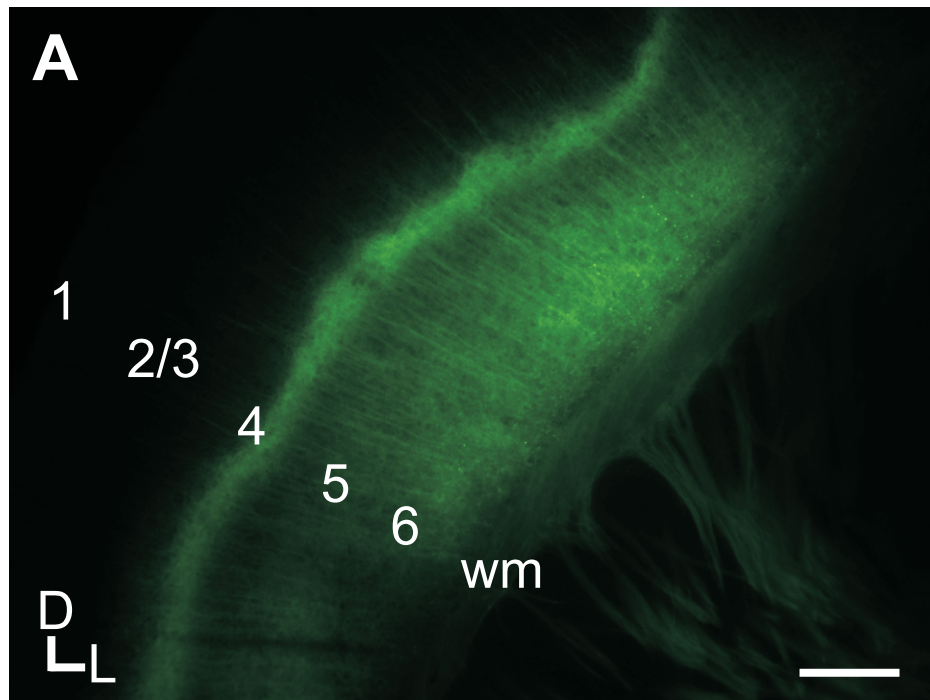
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*Table of Reagents/ Materials Used
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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Polybrene	Santa Cruz Biotech	SC134220	
Paraformaldehyde 32% solution	EMS	15714	
TO-PRO-3 Iodide (1mM solution in DMSO)	Life Technologies	T3605	
Vectashield	Vector Labs	H-1400	
AAV viral constructs	UNC Vector Core	NA	
Mouse stereotactic headholder	Stoelting	51730	
Nanocool injector	Harvard Apparatus	703040	
UV laser: 355 nm, Nd:YVO4; 1 W;	DPSS Lasers, Inc	3500	
Galvonometers	Cambridge scanning	6210	
mirrors	Cambridge scanning	6m2003S-A	
mount	Cambridge scanning	6102103L	
cable	Cambridge scanning	6010-19-120	
driver/controller	Cambridge scanning	67121-1	
Shutter - ZM coated (UV-compatible)	Uniblitz	L23-ZM2	
Shutter Controller	Uniblitz	VMM-D1	
Current Amplifier	Stanford Research Systems	SR570	
6713 board	National Instruments	777741-01	
2110 break-out box	National Instrument	777643-01	
PCI-MIO-16E-4	National Instrument	777383-01	
BNC-2090	National Instrument	777270-01	
Cables	National Instrument	184749-01	
Matlab	Mathworks		
TCS SP2	Leica microsystems		

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Laser-scanning photostimulation of optogenetically targeted forebrain circuits

Author(s):

Charles C. Lee, Ying-Wan Lam, Kazuo Imaizumi, S. Murray Sherman

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AUTHOR:

Name:

Charles C. Lee

Department:

Comparative Biomedical Science

Institution:

School of Veterinary Medicine, Louisiana State University

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Laser-scanning photostimulation of optogenetically targeted forebrain circuits

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Reviewers' comments in bold type

Authors' response in plain type

Editorial comments:

1) Your manuscript has been edited. When addressing future revisions of the document, please use the most current version located in the "File Inventory" of Aries.

We thank the editor for editing the manuscript. As requested, we have revised the document using the most current version located in the "File Inventory".

Reviewers' comments:

Reviewer #1:

This manuscript describes a novel approach to utilize optogenetic neuron-targeting with laser scanning photo stimulation. The revised manuscript seems overall to provide an adequate demonstration of the technique. A few minor clarifications and modifications would further improve accessibility for the reader.

We thank the Reviewer for their comments, which have been used to revise the manuscript. We believe that it has been greatly improved as a result of their guidance.

- A couple of more sentences in the introduction should be added to further point out what is novel about this approach and what can be gained over previous approaches (some of this is already alluded to in the Discussion).

As suggested, we have revised the introduction to highlight the novel aspects of the approach.

- Also in the Introduction one could be a bit more specific about which circuits are being used as examples here.

As suggested, we have explicitly stated which circuits are being used as examples in the manuscript.

- On page 9, two constructs are listed to induce expression of channelrhodopsin. It would be helpful to state which neuron types are specifically targeted by these two approaches (it is clear from the context, but it does not hurt to be explicit here).

We have revised this section to explicitly state the neuronal types targeted by the two approaches, i.e. CaMKIIa promoter driven expression in glutamatergic excitatory neurons and Cre-Lox mediated expression in layer 6 corticothalamic neurons.

- Figure 2 can be improved. 2A had little contrast in my print out and was difficult to read. The impact of Fig 2B, the main result figure demonstrating feasibility, is diminished due to the overlap of the many traces and their 'inelegant' thickness. I would suggest to shift consecutive rows alternately by $\pm \sim 30$ microns. That would allow a better view of the large response peaks at some of the stimulation sites.

We have revised the figure to improve legibility. We were initially constrained by the Journal's mandated 3 pt. line thickness requirement, which has been relaxed by the Editor to accommodate thinner lines for the traces.

Reviewer #2:

Manuscript Summary:

The authors describe their protocol for functional mapping of neural circuits using optogenetics (ChR2) in mice using a floxed AAV construct. The authors show examples of this functional mapping as applied to study the connectivity in V1/V2 and between S1 and VP, measured via laser-scanning photostimulation combined with synaptic physiology and/or confocal microscopy. The results appear to be solid and consistent with the known organization in these areas. The details of the technique are clear and appear to be complete and will be very useful to other investigators to develop this new technology. This manuscript demonstrates remarkable progress in applying this new technology and is an important step towards broadening it

to in-vivo studies and to applying optogenetics in other species.

Major Concerns:

I have no major or minor concerns with the manuscript. The authors appear to have addressed concerns raised in earlier submissions.

We thank the Reviewer for their time in reviewing the manuscript. We appreciate their comments regarding the utility and advances of the methods presented here.

Reviewer #3:

Manuscript Summary:

This paper describes a technique using optogenetics together with laser-scanning photostimulation in order to elucidate functional neuronal circuitry within the slice preparation. The authors injected a viral vector carrying ChR2 in neurons of the mouse forebrain and waited approximately 2-3 weeks for ample ChR2 expression. They then conducted whole-cell patch clamp recordings in neurons in response to ChR2-mediated depolarization at precise locations within the slice. They subsequently use fluorescent imaging of ChR2 expression to help better understand the morphology of the same circuitry.

The method described in this manuscript is broadly applicable, in that future experiments can take advantage of the cell-type-specificity available with ChR2 and use laser-scanning photostimulation to assess EPSCs from various locations within the slice with respect to the postsynaptic neuron of interest. This is a powerful approach. However, enthusiasm for the manuscript would be increased by addressing the following concerns:

We thank the reviewer for their comments, which have been used to revise the manuscript. We believe that it has been greatly improved as a result.

Major Concerns:

1. Perhaps the authors could explain in a bit more detail this interesting technique they are "selling" in the protocol text (they dismiss the technique as "previously described," yet this is a "methods" journal). It would additionally be helpful if there were a schematic figure to help clarify the following:

A) What is being stimulated, and what is being recorded. Imagining the content of the video that will be associated with this paper, making sure that

all readers/viewers are on the same page before viewing the video would make the video more effective.

B) What is the physical arrangement of the stimulation setup, and how is spatially precise photostimulation achieved? It's not clear from the text how light is actually delivered to the slice. Again, understanding this conceptually would make the subsequent viewing of the video more informative. Although this method has been described previously in other publications, this is a methods-oriented paper. Thus, some practical description of the procedures seems appropriate.

We have added a new figure to address these issues that depicts the stimulation setup and the recording and stimulation sites.

2. The authors seem to have nicely addressed most of the previous reviewers' concerns. However, possible limitations to the technique and trouble-shooting suggestions are not listed in the "Discussion" as suggested by reviewer comment #10. For example, how do the authors ensure that light scattering, and therefore consistent effective intensity for ChR2 stimulation, is uniform across the slice? In other words, how do they know that the effective intensity delivered to point A and point B in the slice is the same, given that light may not penetrate the tissue uniformly?

As suggested, we have added a discussion of the uniformity of illumination to the Discussion.

Minor Concerns:

3. The authors repeatedly discuss the cell-type specificity afforded by optogenetics, yet they do not specify what cell type they target in V1 using the CaMKII promoter. Presumably these are excitatory neurons, but it would be worth stating this.

We have revised the Representative Results and the Figure Legend to state explicitly the CaMKIIa promoter driven expression of ChR-EFYP in excitatory glutamatergic neurons.

4. For the example shown in Fig. 3, a transgenic Ntsr1-cre mouse was used in

combination with viral delivery of a floxed ChR2, driven by a ubiquitous promoter (EF1 alpha). Presumably Cre expression in this line of mice is restricted to layer 6 neurons, but again it would be nice to state this in the "Representative results" section and/or provide a reference.

We have clarified the Cre-Lox mediated expression of ChR-EYFP only in layer 6 corticothalamic neurons in both the Representative Results and Figure Legends. In addition, we have added the relevant reference.

5. Given that the benefit of such a cutting-edge technique is to analyze the amplitudes/time courses of postsynaptically-generated current in order to better understand the functional circuitry, some aspects of the figures should be clarified:

A) The representative whole-cell EPSCs in both Fig. 2B and Fig. 3C are difficult to discern, as the traces are too thick/blurry to visualize.

We have revised the figure to improve legibility. We were initially constrained by the Journal's mandated 3 pt. line thickness requirement, which has been relaxed by the Editor to accommodate thinner lines for the traces.

B) Although we understand the relationship between the data in 3C and 3D, it might be helpful to state this in the paper and/or in the figure (e.g., show a box around a trace in 3C and the corresponding pixel in 3D) for the naïve reader.

As suggested, we have boxed the traces and the corresponding pseudocolor plot in the figure.

C) The use of different colors for each row of traces in 3C may also be confusing to a naïve reader, given that these colors do not convey information while the (similar) colors in 3D do.

As suggested, we have adopted a single color (pink) for all of the traces.