Video Article

Mapping Inhibitory Neuronal Circuits by Laser Scanning Photostimulation

Taruna Ikrar[1], Nicholas D. Olivas[1], Yulin Shi[1], Xiangmin Xu[1,2]

[1]Department of Anatomy and Neurobiology, School of Medicine, University of California, Irvine

[2]Department of Biomedical Engineering, School of Engineering, University of California, Irvine

Correspondence to: Xiangmin Xu at xiangmin.xu@uci.edu

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Abstract

Inhibitory neurons are crucial to cortical function. They comprise about 20% of the entire cortical neuronal population and can be further subdivided into diverse subtypes based on their immunochemical, morphological, and physiological properties[1-4]. Although previous research has revealed much about intrinsic properties of individual types of inhibitory neurons, knowledge about their local circuit connections is still relatively limited[3,5,6]. Given that each individual neuron's function is shaped by its excitatory and inhibitory synaptic input within cortical circuits, we have been using laser scanning photostimulation (LSPS) to map local circuit connections to specific inhibitory cell types. Compared to conventional electrical stimulation or glutamate puff stimulation, LSPS has unique advantages allowing for extensive mapping and quantitative analysis of local functional inputs to individually recorded neurons[3,7-9]. Laser photostimulation via glutamate uncaging selectively activates neurons perisomatically, without activating axons of passage or distal dendrites, which ensures a sub-laminar mapping resolution. The sensitivity and efficiency of LSPS for mapping inputs from many stimulation sites over a large region are well suited for cortical circuit analysis.

Here we introduce the technique of LSPS combined with whole-cell patch clamping for local inhibitory circuit mapping. Targeted recordings of specific inhibitory cell types are facilitated by use of transgenic mice expressing green fluorescent proteins (GFP) in limited inhibitory neuron populations in the cortex[3,10], which enables consistent sampling of the targeted cell types and unambiguous identification of the cell types recorded. As for LSPS mapping, we outline the system instrumentation, describe the experimental procedure and data acquisition, and present examples of circuit mapping in mouse primary somatosensory cortex. As illustrated in our experiments, caged glutamate is activated in a spatially restricted region of the brain slice by UV laser photolysis; simultaneous voltage-clamp recordings allow detection of photostimulation-evoked synaptic responses. Maps of either excitatory or inhibitory synaptic input to the targeted neuron are generated by scanning the laser beam to stimulate hundreds of potential presynaptic sites. Thus, LSPS enables the construction of detailed maps of synaptic inputs impinging onto specific types of inhibitory neurons through repeated experiments. Taken together, the photostimulation-based technique offers neuroscientists a powerful tool for determining the functional organization of local cortical circuits.

Video Link

The video component of this article can be found at https://www.jove.com/video/3109/

Protocol

1. Brain slice preparation

1. Transgenic mice are deeply anesthetized with pentobarbital sodium (>100 mg/kg, ip) and rapidly decapitated, and their brains extracted into a frozen and oxygenated cutting solution.
2. GFP goggles are used to visually screen if the mouse brain indeed expresses GFP.
3. Primary somatosensory cortical sections of 400 µm thick are cut with a vibratome in sucrose-containing artificial cerebrospinal fluid (ACSF). Slices are first incubated in sucrose-containing ACSF for 30 min to 1 h at 32°C, and after the initial incubation period, transferred to recording ACSF at room temperature. Throughout the incubation and recording, the slices are continuously bubbled with 95% O_2-5% CO_2.

2. Equipment startup

The overall system implementation is illustrated in Figure 1.

1. The laser cooling system and power supplier is turned on, getting the laser ready.
2. All hardware related to photostimulation control (including the scanning mirror system, an optical modulator, an electronic shutter and a photodiode input amplifier) is turned on.

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3. The electrophysiological equipment (including a Multiclamp 700B amplifier and micromanipulators) is turned on. Electrophysiological recordings, photostimulation, and imaging of the slice preparations are done in a slice perfusion chamber mounted on the motorized stage of the microscope.
4. The imaging camera (Retiga 2000, Q-imaging, Austin, TX) is turned on. Slices are visualized with the upright microscope (BW51X, Olympus) with infrared differential interference contrast (DIC) and epi- fluorescent optics through the imaging system.
5. The Matlab-based EPHUS software and Q-capture camera software are started up. A custom-modified version of Ephus software (Ephus, available at https://openwiki.janelia.org/) is used to control photostimulation protocols and acquire photostimulation data.
6. After the system is on, one should check to see if the laser photostimulation system is at a working state. As the 4x microscope objective is used for delivering the UV flashes for glutamate uncaging, a piece of white paper can be put under to observe the laser scanning pattern while the system runs.

3. Setting up slice perfusion

1. A pressurized perfusion system is turned on to feed the recording ACSF into the slice chamber. Care is taken to ensure a constant fluid level of 2.0 mm above the slice in the chamber.
2. An aliquot of the stock solution of MNI-caged-glutamate is added to 25 ml of circulating ACSF for a concentration of 0.2 mM caged glutamate. Please note that after 5-6 h of experimentation, the bath solution and MNI-glutamate will be refreshed.
3. A brain slice is moved into the recording chamber. One can run the imaging system to check the slice quality and anatomically locate the primary somatosensory area. Then the slice is anchored with a custom-made stringed platinum ring. Be careful not to put the anchor over the brain region intended for recordings.

4. Whole cell patch-clamping recording

1. Glass electrodes (4-6 MΩ resistance) are pulled and filled with an internal solution containing 0.1% biocytin for cell labeling and morphological identification.
2. To perform patch recording, cells are visualized at the 60x objective. Inhibitory cell types are first identified and selected based on visualization of GFP expression under a DIC/fluorescent Olympus microscope; recordings are subsequently performed under visual control aided by infrared DIC video monitoring. Please note that a few times of switching between the DIC and fluorescent modes is necessary to confirm the GFP cell location while approaching the electrode to the target cell.
3. Conventional patch-clamping techniques are applied. The electrode is filled with positive pressure, moved close to the cell surface to establish a discernible dimple upon the targeted cell membrane; and then negative pressure is quickly applied to form a gigaseal and break in while monitoring the current injection responses on the video monitor.
4. Upon breaking in, the images of the cell at 60x under the DIC and fluorescent modes are taken for the on-line verification. Before collection of photostimulation data, hyperpolarizing and depolarizing current pulses are injected to examine each cell's basic electrophysiological properties.
5. Once stable whole cell recordings are achieved with good access resistance (usually <20 MΩ), the microscope objective is switched from 60x to 4x for laser scanning photostimulation. The slice image at 4x is acquired and will be used for guiding and registering photostimulation sites.

5. Laser scanning photostimulation

1. The photostimulation and data acquisition parameters are set by activating the configuration switch module of Ephus. Normally, 1 ms laser (20 mW) with a stimulus interval of 1 second is used for photostimulation mapping. Data traces of 1 second sampled at 10 kHz are acquired.
2. The 4x slice image is loaded into the mapper module of Ephus. The location of cell soma is defined, and photostimulation sites of a 16x16 pattern (80 µm x 80 µm spacing) are set up around the cell location, covering all the cortical layers.
3. The excitation profile of the recorded neuron is mapped by examining the spiking locations in response to photostimulation at the current clamp mode. Our user-friendly software with the on-line display features facilitates the mapping experiments.
4. Local excitatory circuit connections are mapped by detection of excitatory postsynaptic currents (EPSC) from the recorded cell while laser scanning at different locations. The cell is held at -70 mV in voltage clamp mode to detect inward excitatory currents. The EPSC maps are repeated 2-3 times with the photostimulation pattern rotated or flipped.
5. Optional: Local inhibitory circuit connections can also mapped by detection of inhibitory postsynaptic currents (IPSC) from the recorded cell held at close to 0 mV in voltage clamp mode to detect outward inhibitory currents. Note that it is best to use the electrode internal solution with potassium replaced with cesium for IPSC mapping.
6. After all physiological assays are completed, the electrode is gently removed from the recorded cell. The brain slice is taken out and fixed in 4% paraformaldehyde overnight. The recorded cells is stained against biocytin. Cell morphology is examined with confocal or epi- fluorescent microscopy, which also confirms that each recorded cell is indeed the GFP-expressing interneuron originally targeted.

6. Photostimulation data analysis

Our newly developed methodology and software implementation[11] is applied to the detection and measurement of photostimulation-evoked synaptic events in the raw data maps. As exemplified in Figure 2, color-coded maps are constructed to illustrate the pattern of synaptic input to the recorded neuron.

7. Representative Results:

Example results of electrophysiological recording and photostimulation mapping are shown in Figure 2. Targeted recordings of specific inhibitory cell types are made possible by using transgenic mice expressing GFP in known inhibitory cell types. Given the diversity of inhibitory interneurons, analyses of GFP expression, intrinsic electrophysiology and morphological characteristics (Figure 2A) are combined to arrive

at the final cell type classification. Figure 2B-C illustrate that laser scanning photostimulation allows for extensive mapping of local cortical circuit connections to single inhibitory neurons. The raw data map is shown in Figure 2C. The direct uncaging responses are excluded from data analysis (Figure 2C). The quantitative input maps are color-coded, shown in Figure 2D-F. The example fast-spiking inhibitory interneurons received strong excitatory synaptic input (EPSCs) from layer 4 and deeper layers. By relating the cell's synaptic input organization to defined cortical pathways, we are able to infer its possible role (e.g., feedback and feedforward inhibition) in cortical information processing.

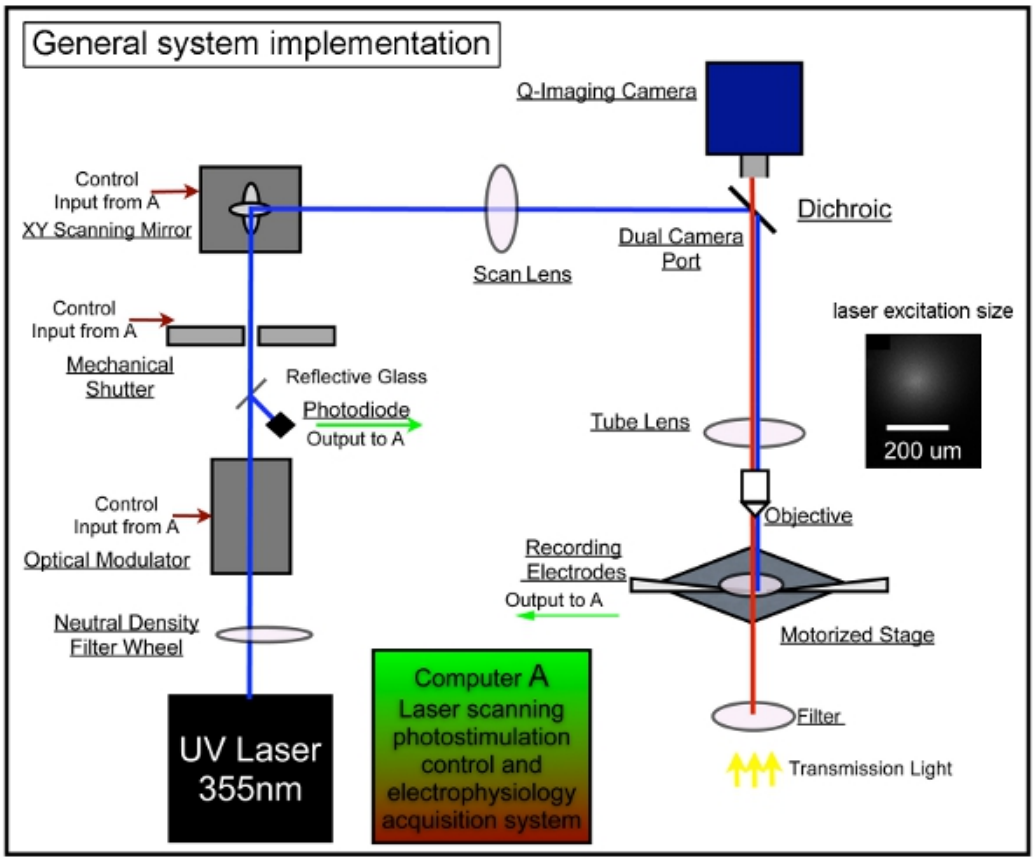

Figure 1. General system instrumentation for laser scanning photostimulation. Our overall system consists of photostimulation control, video imaging, and electrophysiological recording systems. We adopted the design of the LSPS system described previously[7,17]. A laser unit is used to generate 355 nm UV laser for glutamate uncaging. The laser beam is directed through the optical path of our system. Short durations of laser flashes (e.g., 1- 3 ms) are controlled by using an electro-optical modulator (i.e., pockels cell) and a mechanical shutter. Laser beam power is modulated by a neutral density gradient wheel and monitored by diverting a small fraction of the laser beam with a glass coverslip to a photodiode. The laser scanning system includes an X-Y pair of scan mirrors, the scan lens, the tube lens, and the objective lens. The mirrors deliver the laser beam through a scan lens; then the beam enters the microscope via a dichroic mirror and is focused by a custom-made UV-transmitting tube lens. The beam underfills the back aperture of the microscope objective to provide a more columnar (as opposed to conical) illuminating beam, keeping the mapping as two-dimensional as possible by reducing the axial resolution. Under the 4x objective, the laser beam forms uncaging spots, each approximating a Gaussian profile with a width of 153 μm laterally at the focal plane (see the insert). Various laser stimulation positions can be achieved through galvanometers-driven XY scanning mirrors, as the mirrors and the back aperture of the objective are in conjugate planes, translating mirror positions into different scanning locations at the objective lens focal plane. During uncaging, a variable number of patterned sites that covers the whole field are stimulated sequentially in a nonraster, nonrandom sequence to avoid revisiting the vicinity of recently stimulated sites. Please refer to Xu *et al.* (2010) for more detailed information.

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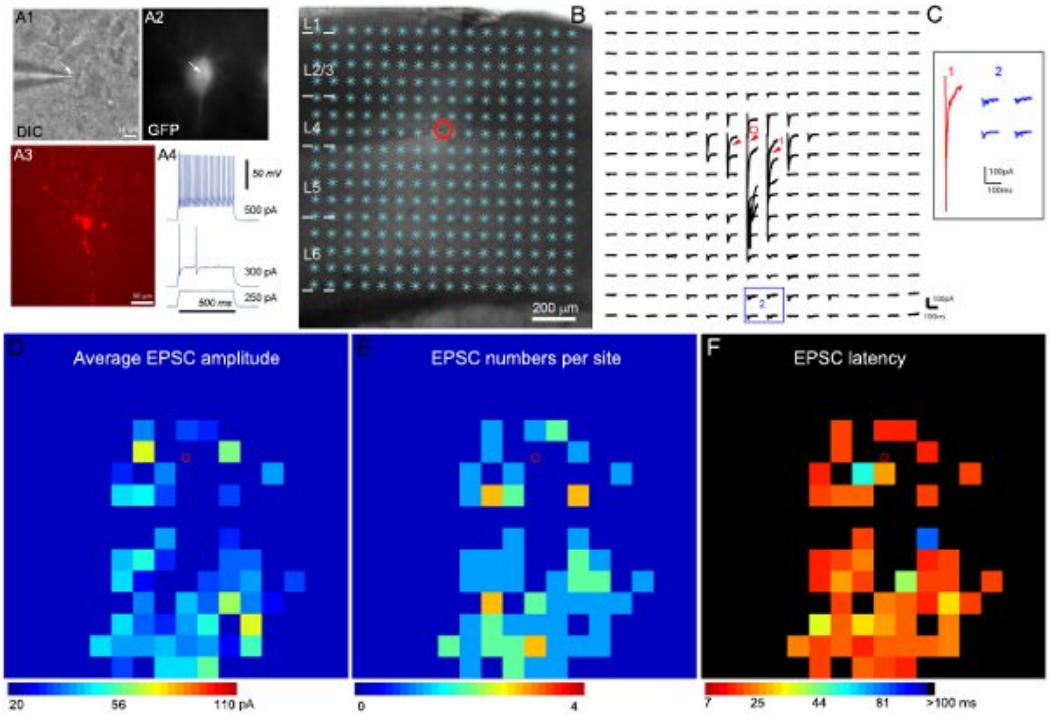

Figure 2. Mapping local excitatory circuit connections to a GFP-expressing fast-spiking interneuron in layer 4 of mouse primary sensory cortex. A1 and A2 show the DIC and GFP fluorescent images of the targeted cell under the 60x objective in the living brain slice, while A3 shows the cell morphology revealed by biocytin staining in the post-recording, fixed slice. A4 shows the firing patterns of the recorded GFP cell, characteristic of a fast-spiking inhibitory interneuron, in response to intrasomatic current injections of different strengths. B show the slice image superimposed with the 16 x16 photostimulation sites (*). The cell location is indicated by the small red circle. C shows an array of photostimulation-evoked response traces from the stimulation locations shown in B, while the cell was held at -70 mV to detect inward excitatory currents. Different forms of photostimulation responses are illustrated by the traces at locations of 1 and 2, which are expanded and separately shown in the insert. The trace 1 is an example of the direct responses (indicated by the red arrowheads) to glutamate uncaging on the cell body and promximal dendrites. Other traces in 2 are typical examples of excitatory synaptic input responses (blue). Direct responses and synaptic inputs can be distinguished by their amplitudes and response latencies. D, E and F are the color-coded maps (16x16 sites) of average EPSC amplitude, the EPSC numbers, and the first detected EPSC latency per site, respectively, for the raw data map shown in C. The average input amplitude from each stimulation site is the mean amplitude of EPSCs in the analysis window, with the baseline spontaneous response subtracted from the photostimulation response of the same site. The number of EPSCs and the arrival time or latency of the first detected EPSC per site are also measured and plotted. Please refer to (Shi *et al.*, 2010) for more details.

Discussion

Photostimulation-based mapping techniques have been effectively applied for analyzing cortical circuits. Laser scanning photostimulation combined with whole cell recording allows high resolution mapping of laminar distributions of presynaptic input sources to single neurons, because the simultaneous recording from a postsynaptic neuron with photostimulation of clusters of presynaptic neurons at many different locations provides quantitative measures of spatial distribution of excitatory or inhibitory inputs. This technique aided by using transgenic mice expressing GFP in known inhibitory cell types has greatly facilitated our current work toward elucidating local inhibitory cortical networks. In the example study, we used caged glutamate to activate neurons in a spatially restrict manner upon laser uncaging. It should be noted that glutamate uncaging can be replaced with photoactivation via channelrhodopsin or other genetically encoded photosensitive molecules in specific cell types[12,13]. Thus the photostimulation technique is possible to target not only specific cortical regions but also specific subset of neurons within their participating circuits[14-16]. In addition, we recently developed a new photostimulation-based mapping technique[9] that incorporates the spatial precision of activation that can be achieved by laser-scanning photostimulation with rapid and high-temporal resolution assessment of evoked network activity that can be achieved by voltage-sensitive dye imaging. Unlike combination of whole cell recordings with photostimulation for mapping local circuit inputs to individually recorded neurons, this innovation can be used to map cortical circuit output and functional connections at the level of neuronal populations.

Disclosures

No conflicts of interest declared.

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