Direct Multiphoton Stimulation of Neurons and Spines

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This chapter describes an optical method to directly stimulate a neuron (i.e., without using caged chemicals) using an infrared ultrafast mode-lock laser. This method can trigger action potentials in a targeted neuron when a laser beam is applied to the somatic membrane. Alternatively, it can mimic excitatory postsynaptic potentials (EPSPs) when applied to dendritic spines.

The protocol has been applied successfully to acute brain slices from the neocortex and hippocampus. It can be used in conjunction with slices bulk-loaded with calcium indicators, such as fura-2AM.

MATERIALS

Two-Photon Microscope

This protocol was optimized using an ultrafast mode-lock (repetition rate 76 Hz, pulse width ~100 fsec) laser beam, produced by a Ti:sapphire laser (Mira 800, Coherent), pumped by a solid-state Nd:YVO$_4$ laser (Verdi-5, Coherent). Similar results were obtained using a Chameleon laser (Coherent). The near-infrared laser beam was collimated and modulated by a Pockels cell (350-50BK, ConOptics), driven by a high-voltage DC power amplifier (Model 302, ConOptics). The output beam was then directed to a laser-scanning system (FluoView, Olympus) coupled to an upright microscope (BX50WI, Olympus). The Pockels cell was modulated by a signal generated using a square-wave generator (Master-8, A.M.P.I.). Transistor-to-transistor logic (TTL) signals marking the laser exposures (LINE ACTIVE and FRAME ACTIVE) were produced by the controller box (FV5-PSU) and used to trigger a square-signal-wave generator. The laser-scanning system and Z-positioning of the microscope objective were controlled by dedicated software supplied by the vendor (FluoView Software, Olympus). A more detailed description of the setup is available elsewhere (Majewska et al. 2000; Nikolenko et al. 2003).

Biological Tissue

The described protocol has been successfully applied using juvenile (postnatal day 7–14) C57 mouse neocortical and hippocampal acute slices (~300 µm thickness).

Fluorescent Indicator

To visualize the target cell, fluorescent indicator was applied either in bulk (fura-2AM) or with the whole-cell patch clamp (e.g., Alexa-488, fura-2, or Calcium Green I, Molecular Probes).

Caution: *See Appendix 3 for appropriate handling of materials marked with <!>.*

PROCEDURE

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1. Prepare acute slices using a standard method. Bulk-load the slice with a fluorescent indicator (e.g., with fura-2AM ester), as described in Chapter 44. Alternatively, label a neuron by intracellular recording (e.g., whole-cell patch-clamp method) with a fluorescent indicator (Chapter 33).

2. Place the slice under the microscope objective (LUMPlanFL/IL 40x [NA = 0.8] or 60x [NA = 0.90], Olympus) and maintain in oxygenated artificial cerebrospinal fluid (125 mM NaCl, 3 mM KCl<!>, 10 mM glucose, 26 mM $NaHCO_3$, 1.1 mM NaH_2PO_4<!>, 2 mM $CaCl_2$<!>, 1 mM $MgSO_4$<!>; pH adjusted to 7.4, ~37°C) superfused through the recording chamber. Hold the slice in the recording chamber by placing a small weight ("harp") consisting of a C-shaped metal piece and a few thin nylon strings, making arcs over the metal piece. Alternatively, allow the slice to stick to the surface of the chamber by briefly (<1 min) draining its perfusate.

3. Scan the slice (xyt scan mode) with minimal laser intensity<!> to visualize fluorescence-loaded cells. Choose a cell to be stimulated (target) from the visualized cell population.

4. Once the target cell has been selected, locate the target cell at the soma level (or the corresponding dendritic spine) by adjusting the z-axis position of the objective using the control software. Mark the pixel coordinates of the cell's location and bring the focal point to the membrane surface by gradually lifting the z-axis position of the objective until the fluorescence signal diminishes.
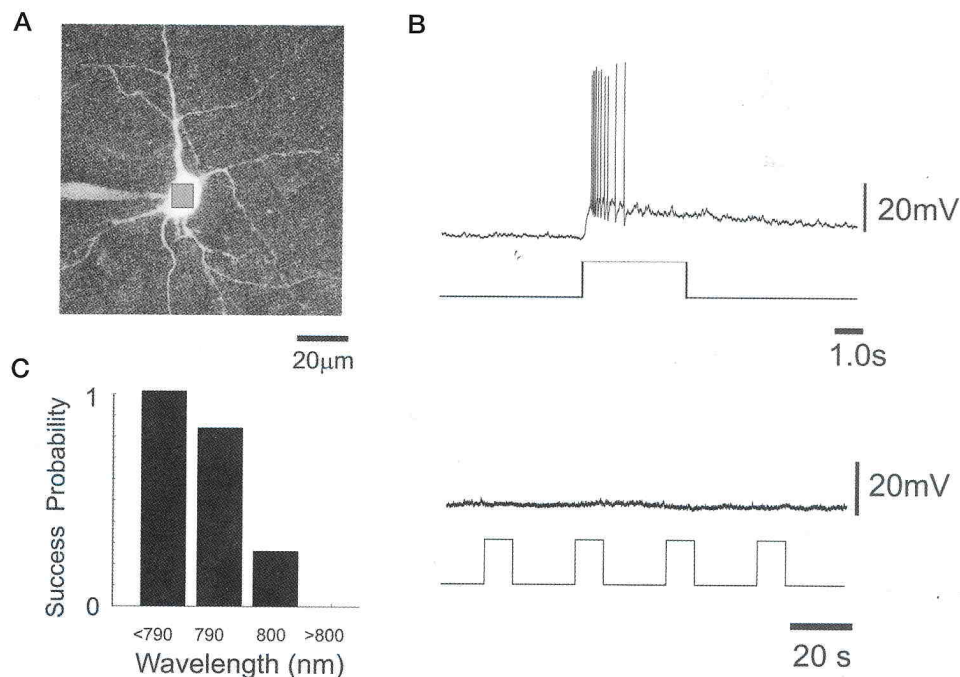

FIGURE 1. Optical stimulation of a cortical layer-5 pyramidal cell. (A) A layer-5 pyramidal cell was labeled with Alexa-488 through a whole-cell patch pipette. Laser irradiation was targeted at the dark square area. (B, Top) When the membrane surface was targeted, the cell was depolarized in response to laser irradiation and produced a burst of action potentials. (Bottom) When the soma was targeted with the laser, no change in membrane potential was recorded. (C) Bar chart indicating the relationship between probability of optical stimulation and excitation wavelength of the mode-lock laser beam. The probability of induction of optical stimulation is dependent on the excitation wavelength. (Modified, with permission, from Hirase et al. 2002 [©Wiley].)

5. Program the modulation signal to the Pockels cell in such a way that a high-intensity (>20 mW at the exit of the objective) excitation beam is applied only in the target cell area, keeping the rest of the scan with minimized laser intensity.

6. Repeated scanning (typically several times) of the membrane surface area of the target cell will lead to gradually increasing depolarizing responses of the target cell, eventually causing it to emit action potentials. Realization of the action potential generation can be monitored optically by calcium imaging of the soma, or electrically via whole-cell intracellular recording or by placing a glass pipette (~1 MΩ) close to the target cell to record the extracellular spikes.

EXAMPLE OF APPLICATION TO SOMATA AND SPINES

To confirm the effectiveness of the stimulation protocol, the membrane potential of the target cell was recorded using the whole-cell patch-clamp method. The cell was fluorescently labeled with Alexa-488 (Molecular Probes). Figure 1 shows an example of direct two-photon stimulation of a neuronal soma.
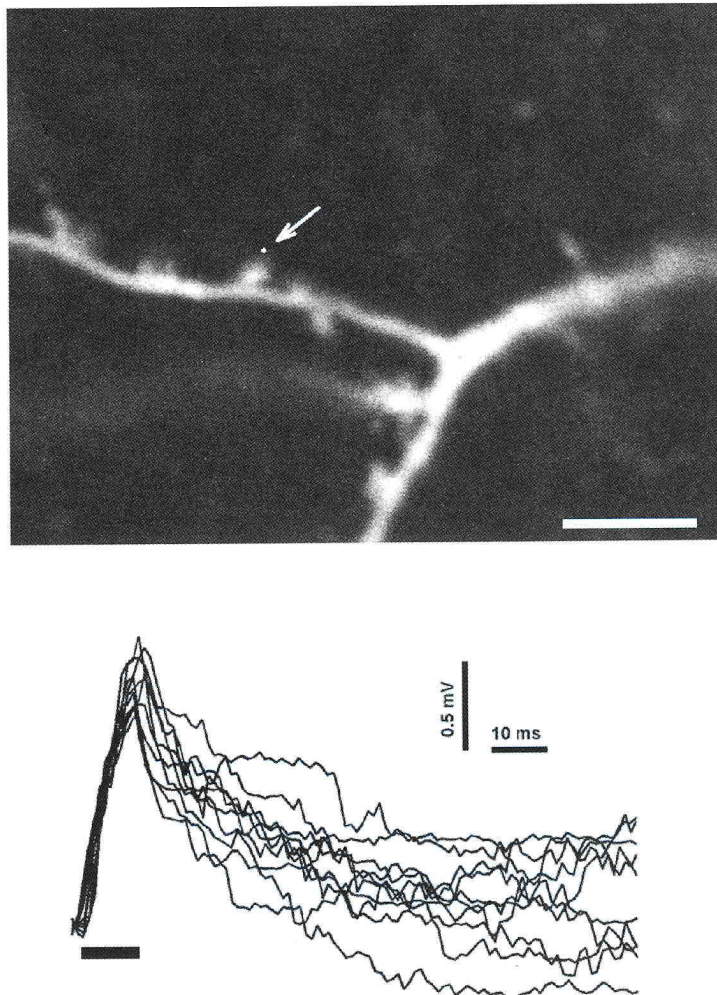

FIGURE 2. Optical stimulation of a spine. Experiment performed in a layer-V large pyramidal neuron from a P13 mouse visual cortex. The neuron was labeled with Alexa-488 (250 μM) using a patch pipette, and the spot above the spine was irradiated with 80–90 mW of average power on the sample (*top panel*). (*Bottom panel*) Simultaneous electrophysiological recording from the soma during the experiment. (*Black bar*) Duration of the irradiation. The laser stimulation produces a depolarizing response that resembles an EPSP. This effect is produced repeatedly.

A neuron was loaded with a fluorescent dye (Alexa 488) through a patch pipette (Fig. 1A). When the laser beam was targeted to the intra-soma area, there was a very small change in the membrane potential (Fig. 1B; bottom trace). In contrast, the membrane potential elicited a depolarizing response to the laser irradiation to the membrane and eventually emitted bursts of action potentials (Fig. 1B; top trace).

The probability of successful optical stimulation depends heavily on the wavelength of excitation (Fig. 1C). Generally, a wavelength of 790 nm or less produces reliable optical stimulation. Wavelengths greater than 800 nm do not produce reliable optical stimulation.

Figure 2 shows an example of a similar experiment, where the stimulated region is next to a dendritic spine (upper panel, spot). Laser irradiation triggers a small depolarization (bottom panel), which resembles the amplitude and kinetics of an EPSP. This optically triggered depolarization can be repeatedly elicited without any significant changes in its amplitude and kinetics. It is possible that the depolarization is due to the activation of the presynaptic terminal impinging on the spine. Regardless of its mechanisms, this method can be used to reliably stimulate any portion of the dendritic tree, without the use of caged compounds.

ADVANTAGES AND LIMITATIONS

An obvious advantage of this protocol is that it does not require the use of any extra chemicals (such as caged compounds) other than fluorescent markers used to label cell populations. This may be particularly important for in vivo experiments, where the application of exogenous chemicals is not straightforward. With recent advances in molecular biology, a wide variety of transgenic mice expressing fluorescent protein in various cell types have become available. These allow a combination of optical stimulation, as described here, and optical probing (Peterlin et al. 2000) to be used to map neuronal connectivity in various kinds of labeled interneurons without electrophysiology or immunocytochemistry. In addition, optical stimulation of spines can be a useful tool to investigate dendritic biophysics. The stimulation regime presented in this chapter works more efficiently for wavelengths shorter than 800 nm. When designing these experiments, it is important that the indicator be effectively excited at the wavelength of the beam used for direct multiphoton excitation (see Chapter 44).

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