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Dual channel photostimulation for independent excitation of two populations

Bryan M. Hooks^{1,2,*}

¹Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, PA

²Office of Naval Research – Reserve Component, Arlington, VA

Abstract

Manipulation of defined neurons using excitatory opsins, including channelrhodopsin, enables studies of connectivity and the functional role of these circuit components in the brain. These techniques are vital in the neocortex, where diverse neurons are intermingled and stimulation of specific cell types is difficult without the spatial, temporal, and genetic control available with optogenetic approaches. Channelrhodopsins are effective for mapping excitatory connectivity from one input type to its target. Attempts to use multiple opsins to simultaneously map multiple inputs face the challenge of partially overlapping light spectra for different opsins. This protocol describes one strategy to independently stimulate two co-mingled inputs in the same brain area to assess convergence and interaction of pathways in neural circuits. This is highly relevant in the neocortex, where pyramidal neurons integrate excitatory inputs from multiple local cell types and long-range corticocortical and thalamocortical projections.

Keywords

Optogenetics; Circuit mapping; Cerebral Cortex; Photostimulation

INTRODUCTION

Manipulation of defined neurons using excitatory opsins, including channelrhodopsin, has enabled studies of connectivity and functional role of these circuit components in the brain. In the neocortex, where diverse neurons are intermingled, stimulation of specific cell types is challenging using conventional stimulation techniques with electrodes, which excite nearby cell bodies and axons. The spatial, temporal, and genetic control available with optogenetic approaches allows selective activation or silencing of defined cell types, useful to study local circuit connectivity of intermingled cell types. Channelrhodopsins are effective for mapping connectivity from one input type to its target. However, using this technique to activate multiple inputs is challenging because different opsin molecules respond to different but partially overlapping light spectra (Klapoetke et al., 2014; Lin et al., 2013; Yizhar et al., 2011). This protocol describes a method using dual-channel optogenetics to independently stimulate two inputs in the same brain area to assess convergence of pathways in neural

*Corresponding author: hooksm@pitt.edu; bryan.hooks@navy.mil.

circuits (Hooks et al., 2015). This is useful in the neocortex, where pyramidal neurons integrate large numbers of excitatory inputs from multiple local cell types and long-range corticocortical and thalamocortical projections (Bock et al., 2011; Cajal, 1995; Douglas and Martin, 2004).

The development of red-shifted channelrhodopsin derivatives (Klapoetke et al., 2014; Lin et al., 2013; Yizhar et al., 2011) suggests the possibility of multiple color stimulation with two excitatory opsins. This multicolor approach has allowed dual use of excitatory and inhibitory opsins (Chow et al., 2010; Chuong et al., 2014; Zhang et al., 2007). Various approaches are possible for simultaneous use of two excitatory opsins using precise light stimulation at different wavelengths (Figure .1). This protocol will describe means to stimulate the red-shifted opsin with stimuli that do not excite the blue-shifted opsin (Hooks et al., 2015). And subsequently excite the blue opsin independently while the red channel is unavailable due to prior depolarization block. In a contrasting approach, a kinetically rapid blue-shifted opsin is excited using a brief pulse (5 ms) of blue light, provide suprathreshold depolarization to one population. Excitation to the red-shifted opsin population is maintained subthreshold by slower kinetics and lower expression level (Klapoetke et al., 2014). This pathway responds to red-shifted illumination, which does not excite the kinetically rapid blue-shifted opsin.

This protocol describes the former approach, useful for studying the convergence of two inputs onto single neurons. Both approaches face challenges in achieving desired control of expression level and stimulation intensity. Distinct cell types will vary in necessary opsin expression level for excitability, and the scope and amplitude of light-based stimulation will depend on the tissue targeted and the means to gain optical access through transgenic or virally-encoded expression in mice.

Surgical and experimental procedures described in this protocol follow the National Institutes of Health guidelines for mice and were approved by the Institutional Animal Care and Use Committees of University of Pittsburgh and Janelia Research Campus. Work with viral vectors to express opsins requires approval of Institutional Biosafety Committees (IBC). For Adeno-Associated Virus (AAV), work at Biosafety Level 1 or 2 is appropriate, depending on the guidelines of the IBC at each institution.

Basic Protocol 1 DUAL CHANNEL PHOTOSTIMULATION USING CHR2 AND REACHR

The convergence of two axon populations onto single neurons is studied by first expressing a blue-shifted (ChR2) and a red-shifted (ReaChR) opsin in each of the two putative presynaptic cell types. Brain slices are prepared in target areas where the two axon populations overlap, and whole cell recordings made from potential postsynaptic neurons. Presynaptic axon populations are stimulated as described below.

Materials

Wildtype or transgenic mice—Adeno-associated virus to express ChR2 (Boyden et al., 2005; Nagel et al., 2003; Petreanu et al., 2007; Petreanu et al., 2009) and ReaChR (Hooks et al., 2015; Lin et al., 2013)

Leica VT1200S vibratome (or equivalent) for slice preparation

Olympus BX50WI/ BX51WI microscope (or equivalent) with moveable stage for slice recording

Cairn OptoLED illumination system with 470 nm, 565 nm, and 590 nm LEDs (longer wavelengths may be considered including 617 nm)

Optical elements including filters and dichroics (Chroma; see Figure .2)

Axopatch 700B amplifier (or equivalent) for whole-cell recording

1. Express ChR2 and ReaChR in two distinct pathways in mouse brain. Stereotaxic injection of AAV is the most commonly used method for expression of channelrhodopsins in mouse brain.
2. Stereotaxic surgery in mice can be done under isoflurane anaesthesia, with injection of buprenorphine (0.1mg/kg). Suggested medication for post-operative pain management are Ketoprofen (7–10 mg/kg) or Carprofen (5–10 mg; available as a dietary gel). Briefly, mice are anaesthetized with isoflurane and placed in the stereotax. Once the skull is exposed and bregma is identified, a small burr hole can be drilled in the appropriate location for viral injection. Following viral injection, the skin over the skull is sutured and the animal allowed to recover on a heated pad until ambulatory. Ketoprofen injection is given at this time.
3. AAV injections are typically given at a titer of 1E12 to 9E13 GC/ml. AAV with a range of viral serotypes is available at commercial vector cores. For cortical injections, injection volumes of 30–50 nL per injection site (with separate injections at 0.5 and 0.8 mm in a given penetration) are recommended. A single thalamic injection of 30–50 nL is recommended. Larger injections are possible (of up to 1 μ L), though non-specific effects are a concern (Jackman et al., 2014). The pipette is left in place in the penetration for 5 minutes following injection (or at the end of all injections, if multiple injections are performed along a cortical penetration). AAV is stored at –80 C in small aliquots (~3 μ L) such that 1 μ L can be loaded at a time and freeze-thaw cycles minimized.
4. To study the projections of both neural populations in whole cell recording, the axons of both projections must overlap in a region of interest. This can most readily be accomplished by injecting AAV expressing ChR2-tdTomato (Addgene 28017) and ReaChR-mCitrine (Addgene 50954) into two cortical or thalamic areas that project to the same target area. For this study, primary motor cortex (M1) was used as the target, and primary somatosensory cortex (S1) and posterior thalamus (PO) were used as the presynaptic areas. Experiments studying the properties of the channels alone were conducted in S1. The stereotaxic coordinates for these sites are (in mm):
 - a. Primary somatosensory cortex – barrel region (S1): –0.6 posterior to bregma, 3.0 lateral, 0.5–0.8 depth

- b. Posterior thalamus (PO): −1.2 posterior to bregma, 1.2 lateral, 2.75 depth
 - c. Primary motor cortex – whisker region (M1): +1.2 anterior to bregma, 1.0 lateral, 0.55–0.85 depth
- 5. If AAV transfection is not desired, similar approaches could be conducted by using transgenic mice such as Ai32 (RCL-ChR2(H134R)-EYFP (Jax 012569), Ai27 (RCL-ChR2(H134R)-tdT (Jax 012567), or Rosa26-CAG-LSL-ReaChR-mCit (Jax 026294) (Hooks et al., 2015; Madisen et al., 2012). Transgenic approaches may result in lower but more uniform expression levels. Stimulation protocols are expected to be the same (Hooks et al., 2015), with the opsin expressed transgenically instead of virally.
- 6. Following sufficient time for opsin expression (>2 weeks), prepare living 300 μm coronal brain slices for stimulation and recording (Hooks et al., 2013; Weiler et al., 2008). 300 μm is an optimal thickness such that neurons in the middle of the slice (~100 μm deep) can be visualized for patching in a slice that is sufficiently thick to minimize cutting of dendrites in cells far from the slice surface and maintain apical dendrites of larger pyramidal neurons intact. Anaesthetize animals with isoflurane and decapitated. Rapidly remove brains and place in chilled choline-based cutting solution (see Reagents and Solutions and Hooks et al., 2013; Weiler et al., 2008). Prepare 300 μm coronal brain slices on a Leica VT1200S vibratome. Slices recover for 30 minutes in artificial cerebrospinal fluid (ACSF) at elevated temperature (37 C) prior to recording.
- 7. Prepare microscope for optical stimulation as outlined in this step. Generally, stimulation will occur on a slice electrophysiology rig. Collimated LEDs make ideal light sources for full field stimulation. Pulses which control gain of LED intensity should be prepared and the light intensity of each LED (470 nm and 590 nm) should be measured at the specimen place. This is performed by passing LED light through an aperture of known area (mm^2) and measuring power (mW) with a laser power meter, giving LED intensity in mW/mm^2 . LED illumination should appear uniform throughout the plane of stimulation. Otherwise, reperform the collimation step for the LED. LED intensity will vary depending on the objective used (switching objective from high to low power will reduce the intensity).
- 8. LED gain must be set to match illumination. This was done to match photon flux ($\Phi/\text{sec} \cdot \text{mm}^2$). It is possible that results will be similar if LED intensity (mW/mm^2) is directly matched, though this was not tested. Since the energy of each photon is determined by the wavelength of light, $E_{\text{ph}} = hc/\lambda$, the relative intensity needed for 470 nm and 590 nm varies. Thus, $E_{\text{ph-470nm}}/E_{\text{ph-590nm}} = \lambda_{590\text{nm}}/\lambda_{470\text{nm}}$. (This also is the basis for the offset in Intensity between 590 nm and 470 nm stimulation shown in, for example, Figure .3G.). These intensities might be adjusted in unison on different sweeps (e.g. both at 10%, 20%, or 50% of maximal).

9. Perform whole cell recording of visually identified neurons in regions where there is substantial overlap of afferents from both pathways. Using a fluorescence microscope and oxygenated ACSF, evaluate axonal fluorescence of both pathways to record input in a region of M1 where there is substantial axonal (mCitrine and tdTomato) fluorescence overlap. In the examples shown here, recordings will be performed in M1 pyramidal neurons in voltage clamp at -70 mV holding potential). Expected synaptic inputs (from S1 or PO) are glutamatergic and evoke inward current at -70 mV for typical synapses using K-gluconate-based internal solution and standard ACSF with 2 mM Ca^{2+} and 1 mM Mg^{2+} .
10. Optically stimulate the slice preparation using a known, matched LED intensity (Step 4). A typical stimulation paradigm can be executed as follows:
 - a. The first sweep will include 100 ms baseline, followed by 50 ms LED stimulation at 590 nm. This is immediately followed by 50 ms LED stimulation at 470 nm. This will first result in excitation of the red-shifted opsin pathway (expressing ReaChR-mCitrine in S1, for example) at onset of 590 nm illumination. Excitation of the blue-shifted opsin pathway (expressing ChR2-tdTomato in S1, for example) will follow at onset of 470 nm illumination. Stimuli shorter than 50 ms may be considered, but will require additional testing. The minimum delay time is governed by the slower onset kinetics of the red-shifted opsin.
 - b. The second sweep will include 100 ms baseline, followed by 100 ms LED stimulation at 590 nm. This is immediately followed by 50 ms LED stimulation at 470 nm. This will first result in excitation of the red-shifted opsin pathway (expressing ReaChR-mCitrine in S1, for example) at onset of 590 nm illumination. Excitation of the blue-shifted opsin pathway (expressing ChR2-tdTomato in S1, for example) will follow at onset of 470 nm illumination. This paradigm should be effective with a range of durations for the initial 590 nm stimulation. Testing this with various duration 590 nm pulses for shorter or longer periods of time (50 ms, 100 ms, and 250 ms in step 6.c) will confirm that there is no subthreshold activation of the blue-shifted channel, as evidenced by no change in EPSC amplitude evoked by 470 nm illumination.
 - c. Third sweep will include 100 ms baseline, followed by 250 ms LED stimulation with 590 nm LED. This is immediately followed by 50 ms LED stimulation with 470 nm LED. This will first result in excitation of the red-shifted opsin pathway (expressing ReaChR-mCitrine in S1, for example) at onset of 590 nm illumination. Excitation of the blue-shifted opsin pathway (expressing ChR2-tdTomato in S1, for example) will follow at onset of 470 nm illumination. The longer illumination with 590 nm is used to confirm that there is no subthreshold activation of the blue-shifted channel, as evidenced by no change in EPSC amplitude.

The varying delay times (50, 100, and 250 ms) are arbitrary and used only to confirm no crosstalk exists between 590 nm LED and the blue-shifted channel. Delay times up to 10 s were tested. The benefit of testing in each experiment with a range of delay times is the confirmation that there is no subthreshold activation of the blue-shifted channel, as measured by the absence of change in EPSC amplitude following 470 nm illumination.

- d. Fourth and fifth sweeps of 50 ms illumination with either 590 nm or 470 nm LED alone are also possible to test amplitude of individual activation of red-shifted responses alone, or both together.

REAGENTS AND SOLUTIONS

Choline-based cutting solution (in mM):

110 choline chloride

3.1 sodium pyruvate

11.6 sodium ascorbate

25 NaHCO₃

25 D-glucose

7 MgCl₂

2.5 KCl

1.25 NaH₂PO₄

0.5 CaCl₂

Artificial cerebrospinal fluid (ACSF; in mM):

127 NaCl

25 NaHCO₃

25 D-glucose

2.5 KCl

2 CaCl₂

1 MgCl₂

1.25 NaH₂PO₄

Potassium gluconate-based internal solution (in mM; pH 7.27; 287 mOsm):

128 potassium gluconate

4 MgCl_2

10 HEPES

1 EGTA

4 Na_2ATP

0.4 Na_2GTP

10 sodium phosphocreatine

3 sodium L-ascorbate

Dyes may be added (3 mg/mL biocytin/neurobiotin; 20 μM Alexa-488/594)

pH is adjusted with KOH or HCl.

COMMENTARY

Background Information

Optical activation using channelrhodopsins is a rapidly evolving field. A wide range of molecules exist for excitation and inhibition (Mattis et al., 2012). These might be used in a large number of combinations (for example, to co-express inhibitory opsins with excitatory ones; Chuong et al., 2014). Thus, the range of potential techniques is quite large. The approaches described here are effective for the opsin pairs, illumination conditions, and cell types discussed and are amenable to adaptations to new technologies and experimental questions. Given rapid changes in the field, the question of the investigator, and new technologies available to address it should be considered when selecting an approach.

Critical Parameters

Red illumination must not activate the blue-shifted opsin—To isolate input sources, it is critical that red-shifted activation of ReaChR does not activate ChR2. If ChR2 were activated by 590 nm stimulation, even to a subthreshold degree, this would undermine the independence of the two stimuli. Therefore, it is important to confirm that the stimulus paradigm does not activate ChR2 at 590 nm. Figure .1 A-B illustrate typical spectra for a range of channelrhodopsins and LEDs that might be used for stimulation. The relatively broad activation spectra make selection of light appropriate for avoiding ChR2 activation a challenge. Figure .1D illustrates the intended plan: to activate the red-shifted opsin with long wavelength light (>590nm), while activating both opsins with short wavelength light (<470 nm). Because ChR2 responses are possible at longer wavelengths (up to ~585 nm), testing is required to ensure independent activation of ReaChR at these longer wavelengths. As will be shown, subtraction of two responses is not necessary to quantify the response. Selection of 590 nm wavelength with optical filtration of <585 nm wavelengths for excitation was

empirically determined to independently activate ReaChR. Alternative wavelengths are possible with other opsin pairs.

Figure .3A-C demonstrates the direct responses of ChR2 (A) and ReaChR (B) to long duration pulses at a range of stimulus intensities from 470 nm and 590 nm LEDs. For the same photon flux, 470 nm and 590 nm responses are similar for ReaChR, but ChR2 response is reduced to zero at 590 nm stimulation. It is notable that the response kinetics of ChR2 and ReaChR are quite different. ReaChR rise and fall kinetics are much slower (Figure .3D-G), thus suggesting other strategies to separate stimulation by relying on kinetically fast blue-shifted responses to short duration pulses. The difference in time to peak, for example, could be exploited along with differences in expression level for stimulation of blue-shifted opsins alone, assuming tight control of ReaChR and ChR2 expression (or other opsins). This approach is explored in more detail elsewhere (Klapoetke et al., 2014).

Red illumination must activate the red-shifted opsin—The absence of ChR2 response to 590 nm stimulation was the result of shifting excitation to longer wavelengths. 565 nm, 590 nm, 617 nm, and 627 nm LED stimulation were attempted. 565 nm LED illumination does effectively activate ChR2 (Figure .1A). 590 nm LED illumination did not readily activate ChR2 upon cursory examination, but in fact caused subthreshold depolarization. ChR2 synaptic responses to 470 nm following 590 nm became smaller depending on the length of prior illumination with 590 nm LED. Inspecting direct ChR2 responses in more detail revealed a small current (~5–10 pA, Figure .2A), which reflected <5% of the maximal ChR2 current. This could be abolished by filtering the 590 nm LED output (Figure .2B) with a bandpass (D607/45, Chroma) or long pass (ET 590 LP, Chroma) filter, eliminating small 590 nm-evoked ChR2 currents. Shifting excitation to further red wavelengths (617 nm and 627 nm LED) was tested, but ReaChR+ axons failed to produce detectable synaptic currents in response to these LEDs. Examination of the direct response kinetics (Figure .4) suggested that the rise time of ReaChR in response to long wavelengths was too slow to produce an action potential in severed axons. Previous characterization of ReaChR is consistent with slowed channel kinetics with longer wavelengths (Lin et al., 2013). This does not however, rule out use of these LEDs with other red-shifted opsins (Klapoetke et al., 2014). Furthermore, longer wavelengths may be more effective at successful activation in other cell types where kinetically slower activation is still capable of eliciting an action potential (for example, spinal cord).

Prolonged illumination must evoke single action potentials in axons or boutons—Having one channel that is activated exclusively by red-shifted light makes it possible to excite that pathway by initial illumination with the 590 nm LED. However, achieving independent stimulation of the second channel with blue light is a challenge since this stimulus can excite both the red- and blue-shifted opsins. The experimental design calls for initial stimulation with 590 nm LED capable of evoking a single action potential from a given pathway, followed by subsequent 470 nm stimulus. The neuron type expressing red-shifted opsin does not fire multiple action potentials (APs) but instead enters depolarization block during prolonged illumination, while the pathway expressing blue-shifted opsin is

available to fire at the later time point when the 470 nm LED turns on. Thus, sequential stimulation that exploits the cellular property of depolarization block will enable independent excitation of the two inputs.

How opsin-positive axons respond to prolonged photocurrents was explored as follows. First, whole-cell recordings were made from Layer 2/3 (L2/3) pyramidal neurons in acute slice of primary somatosensory cortex (S1) expressing ChR2 following viral injection. In current clamp mode, 500 ms depolarizing current injections were made to evoke trains of APs (Figure .5A). Typically, pyramidal neurons can fire accommodating trains of APs. The train is longer and more sustained at near threshold levels of injected current, while more substantial depolarization results following greater magnitudes of current injection. Responses to current injection vary widely across cell types (Zeng and Sanes, 2017), so this may be one factor in determining the effectiveness of this stimulus paradigm.

Over-bouton or axonal stimulation might result in a similar pattern of firing APs followed by silence, here called depolarization block. LED stimulation over axons (Figure .5B-D) indeed evoked one action potentials per stimulus. This measurement is limited, since it represents monitoring of back-propagating action potentials instead of a more direct measurement of axonal (or axon terminal) membrane potential, which was not attempted due to technical limitations. This suggested that, for cortical pyramidal neurons, sustained photostimulation of axons or presynaptic boutons would evoke a single presynaptic AP followed by depolarization block.

In some trials with longer 590 nm illumination, a persistent current was noted during the delay between 590 nm and 470 nm illumination. This persistent current was proportional to the initial synaptic current and represented a relatively small fraction of the strength of synaptic transmission. Although earlier controls had suggested that axons did not repeatedly fire APs, the presynaptic terminals might be persistently depolarized by red-shifted opsin stimulation, if opsin expression were sufficiently high in presynaptic boutons, and such local depolarization were sufficient to evoke synaptic release in the absence of evoked APs. To characterize the source of the persistent current, it was studied under different expression levels of ReaChR. Expression level was varied in these experiments by diluting AAV-Syn-ReaChR-mCitrine injections from undiluted (1/1) to 1/9 to 1/50 dilution and comparing the remaining persistent current during prolonged (1 s) photostimulation with 590 nm LED (or 470 nm LED for ChR2). The persistent current was related to the degree of AAV dilution, and was reduced to $\sim 0.067 \pm 0.009$ of the peak current amplitude for the 1/50 dilution. In comparison, persistent current during prolonged ChR2 stimulation was 0.018 ± 0.008 of the normalized peak amplitude (Figure .6A-B). This current may derive from synaptic release driven by direct ReaChR/ChR2-mediated depolarization of the axon terminal (over-bouton stimulation). Blocking voltage gated sodium channels (VGSCs) and AP conduction with TTX did not eliminate the persistent current, but did reduce it to $\sim 70\%$ of maximum. Consistent with a slower, non-VGSC dependent mechanism, onset of the persistent response is slower in TTX. Thus, it is recommended to perform dual channel experiments using low viral titers to avoid overexpression of opsins.

Since this dual channel photostimulation paradigm produces depolarization block in the pyramidal neuron axon, it is possible that this approach would be useful in some but not all neuron populations to achieve depolarization block during sustained ReaChR activation could occur. Medium spiny neurons in striatum receive input from M1 and S1 neurons (Wall et al., 2013), though it has only recently been demonstrated that these axons innervate the same individual neurons (Hooks, 2018). Figure .7A-C demonstrates the effectiveness of the dual channel photostimulation approach for striatal branches of cortical axons. However, this approach was ineffective for studying cortical interneuron inputs to pyramidal cells due to the absence of depolarization block in the presynaptic neurons. In S1, somatostatin-positive interneurons expressing ChR2 fire repeatedly in response to prolonged stimulation (Figure .7D-F), resulting in a sustained IPSC in L5B pyramidal neurons in S1. These neurons are capable of sustained firing at high rates (Urban-Ciecko and Barth, 2016), which might explain the failure to enter depolarization block. Differences in response might also occur for stimulation over somata or axons alone in contrast to over-bouton stimulation. Thus, it is necessary to test neuron populations individually to determine whether this optical stimulation paradigm is suitable.

Troubleshooting

A wide range of LED light sources and excitatory opsins for stimulation are available (Figure .1). Thus, for this approach to be effective in achieving independent photostimulation of each channel, it is ideal for each investigator to confirm the lack of interaction between the red-shifted and blue-shifted pathways during responses to red or blue light on their equipment at their chosen parameters.

Subthreshold activation of the blue opsin during red light stimulation can be quantified. First, a direct measurement of blue-shifted opsin response during red-shifted excitation is possible (Figure .2). Other predictions of subthreshold blue response during orange excitation might be detected by examining blue responses during prolonged orange illumination. If an opsin were excited, even at a subthreshold level, then a smaller response to photostimulation might result when presented with a subsequent, more intense stimulus. Prior activation might reduce the number of opsin molecules available for activation as well as alter the activation/inactivation state of voltage gated sodium and potassium channels. In this case, the blue-shifted opsin response might be smaller – and thus less likely to cause an AP – if a subset of the opsin was already activated due to a prior stimulus. The way this is detected in the paradigm is as follows. Stimulate the preparation on different sweeps with red-shifted (590nm) pulses of varying lengths (Basic Protocol Step 6a-d). If the blue-shifted opsin response is independent of the duration of red-shifted illumination, then subthreshold responses have been eliminated (Figure .8C).

When extending this paradigm to other cell types, it is important to know whether the firing pattern of the proposed presynaptic neuron will result in depolarization block or continuous firing. This might be tested by making whole cell recordings from the proposed presynaptic cell type and verifying via current injection that the axon can enter depolarization block (Figure .5A) or that the axon in response to photostimulation does, in fact, fire only one AP (Figure .8C-D).

Understanding Results

Interpreting circuit mapping data is complicated since stimulation paradigms often excite multiple axons from a given population. There are approaches proposed to study unitary synaptic connection amplitude using optical methods (Morgenstern et al., 2016), though in most preparations multiple axons are simultaneously activated. The number of opsin-positive axons varies between individual animal injections. Thus, in quantitative experiments, it is advantageous to normalize response amplitude within a given animal. Furthermore, the laminar nature of cortex permits normalization of responses across layers (Hooks et al., 2013; Petreanu et al., 2009).

How to perform this normalization to quantify input from two pathways is a fair question. In cortex, it is possible to record in visually identified areas where the two pathways overlap. In cases where the axonal overlap can be visually identified, it can be stated with more confidence whether a certain individual neuron gets input from one or both pathways. It is recommended that such experiments include in each preparation recordings from a cell type that does get input from each of the opsin-positive pathways as a positive control that both have sufficient expression level to permit photostimulation.

A second complicating factor in interpreting results is identifying what the magnitude of the response means. In preliminary experiments, it was proposed that, since means existed to study both the red-shifted pathway alone (with 590 nm stimulation) and both red-shifted and blue-shifted pathways together (with 470 nm stimulation), that the response from the blue-shifted pathway could be quantified by subtraction alone. However, a comparison of the magnitude of the blue-shifted synaptic response following 250 ms delay to the subtraction of 590 nm responses from the combined response revealed that the combined response is ~1.5x the expected magnitude by simple addition (Figure .9). This nonlinear response of the combined response relative to each pathway alone was suggested to be due to loss of voltage clamp in dendrites or spines (Beaulieu-Laroche and Harnett, 2018). It would be interesting to test this in current clamp mode to see if the same result occurs. For voltage clamp recordings, however, variability evident in individual trials (a ratio ~0.7 to ~5) suggested that applying a uniform correction factor for this imperfect measurement would not suffice.

Time Considerations

Opsin expression is critical for this protocol to succeed. One prevalent way to express opsins for long-range circuit mapping is via AAV-mediated transfection (Petreanu et al., 2009). Expression with promoters including Syn, CAG, and CaMKII α generally require ~2 weeks for expression at sufficient levels such that opsins have trafficked into axons at sufficiently high levels to render them excitable. Experimentation with the specific AAV serotype and promoter is worthwhile for targeting specific cell types and optimizing expression. Depending on expertise, animals may be stereotactically injected at postnatal day P11-P14, though some groups are able to inject at early as P1-P3 for brain regions that are easier to target. Thus, experiments may be performed ~2 weeks later. In some cortical injection cases, AAV2/1-Syn-ReaChR-mCitrine was effective for axonal excitation as early as 7 days post-injection.

Other viral approach approaches, including lentivirus (Aronoff and Petersen, 2006, 2008) and rabies virus (Kiritani et al., 2012; Wickersham et al., 2010), are also effective. *In utero* electroporation is also a particularly specialized way to target specific laminae of cortical pyramidal neurons, and results in strong ChR2 expression (Petreanu et al., 2007). Timing for IUEP requires an *in utero* surgery (~embryonic day E18), but expression of opsins transfected at that age will express at high levels and be useful for circuit mapping earlier in development.

Alternatively, expression could be controlled by the use of transgenic mice with the opsin such as ChR2 or ReaChR, inserted as a transgene (Hooks et al., 2015; Madisen et al., 2015; Madisen et al., 2012). These would be expressed at sufficient levels for excitation during circuit mapping as soon as ~2 weeks after Cre- or Flp- expression. The advantage of transgenic approaches vis-à-vis virally-driven expression is that opsin expression is far more uniform (see Figure 1C, Hooks et al., 2015). Uniform expression levels likely confer more uniform responses following photostimulation, and thus might be advantageous for keeping responses subthreshold or suprathreshold for entire axon populations in response to a defined photostimulus (Klapoetke et al., 2014).

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LITERATURE CITED

- Aronoff R, and Petersen CC (2006). Controlled and localized genetic manipulation in the brain. *J Cell Mol Med* 10, 333–352. [PubMed: 16796803]
- Aronoff R, and Petersen CC (2008). Layer, column and cell-type specific genetic manipulation in mouse barrel cortex. *Front Neurosci* 2, 64–71. [PubMed: 18982108]
- Beaulieu-Laroche L, and Harnett MT (2018). Dendritic Spines Prevent Synaptic Voltage Clamp. *Neuron* 97, 75–82 e73. [PubMed: 29249288]
- Bock DD, Lee WC, Kerlin AM, Andermann ML, Hood G, Wetzel AW, Yurgenson S, Soucy ER, Kim HS, and Reid RC (2011). Network anatomy and in vivo physiology of visual cortical neurons. *Nature* 471, 177–182. [PubMed: 21390124]
- Boyden ES, Zhang F, Bamberg E, Nagel G, and Deisseroth K (2005). Millisecond-timescale, genetically targeted optical control of neural activity. *Nature neuroscience* 8, 1263–1268. [PubMed: 16116447]
- Cajal S.R.y. (1995). *Histology of the Nervous System* (New York: Oxford).
- Chow BY, Han X, Dobry AS, Qian X, Chuong AS, Li M, Henninger MA, Belfort GM, Lin Y, Monahan PE, et al. (2010). High-performance genetically targetable optical neural silencing by light-driven proton pumps. *Nature* 463, 98–102. [PubMed: 20054397]
- Chuong AS, Miri ML, Busskamp V, Matthews GA, Acker LC, Sorensen AT, Young A, Klapoetke NC, Henninger MA, Kodandaramaiah SB, et al. (2014). Noninvasive optical inhibition with a red-shifted microbial rhodopsin. *Nature neuroscience* 17, 1123–1129. [PubMed: 24997763]
- Douglas RJ, and Martin KA (2004). Neuronal circuits of the neocortex. *Annu Rev Neurosci* 27, 419–451. [PubMed: 15217339]
- Hooks BM, Lin JY, Guo C, and Svoboda K (2015). Dual-Channel Circuit Mapping Reveals Sensorimotor Convergence in the Primary Motor Cortex. *J Neurosci* 35, 4418–4426. [PubMed: 25762684] Demonstration of two channel photostimulation using depolarization block. Development of transgenic red-shifted ReaChR mouse whose expression is tightly regulated,

sufficient for optogenetic excitation, and dependent on Flp recombinase, Cre recombinase, or both recombinases for expression.

- Hooks BM, Mao T, Gutnisky DA, Yamawaki N, Svoboda K, and Shepherd GM (2013). Organization of cortical and thalamic input to pyramidal neurons in mouse motor cortex. *J Neurosci* 33, 748–760. [PubMed: 23303952]
- Hooks BM P. AE; Paletzki R; Feroze M; Eastwood BS; Couey JJ; Winnubst J; Chandrashekar J; Gerfen CR (2018). Cell type-specific variation of somatotopic precision across corticostriatal projections. *bioRxiv*. 10.1101/261446
- Jackman SL, Beneduce BM, Drew IR, and Regehr WG (2014). Achieving high-frequency optical control of synaptic transmission. *J Neurosci* 34, 7704–7714. [PubMed: 24872574]
- Kiritani T, Wickersham IR, Seung HS, and Shepherd GM (2012). Hierarchical connectivity and connection-specific dynamics in the corticospinal-corticostriatal microcircuit in mouse motor cortex. *J Neurosci* 32, 4992–5001. [PubMed: 22492054]
- Klapoetke NC, Murata Y, Kim SS, Pulver SR, Birdsey-Benson A, Cho YK, Morimoto TK, Chuong AS, Carpenter EJ, Tian Z, et al. (2014). Independent optical excitation of distinct neural populations. *Nat Methods* 11, 338–346. [PubMed: 24509633] Demonstration of two channel photostimulation using subthreshold (for red-shifted opsins) and suprathreshold blue light stimuli (for kinetically rapid blue-shifted opsins). Development of a red-shifted channelrhodopsin, Chrimson, and a blue-shifted channelrhodopsin, Chronos.
- Lin JY, Knutsen PM, Muller A, Kleinfeld D, and Tsien RY (2013). ReaChR: a red-shifted variant of channelrhodopsin enables deep transcranial optogenetic excitation. *Nature neuroscience* 16, 1499–1508. [PubMed: 23995068] Development of a red shifted channelrhodopsin, ReaChR.
- Madisen L, Garner AR, Shimaoka D, Chuong AS, Klapoetke NC, Li L, van der Bourg A, Niino Y, Egolf L, Monetti C, et al. (2015). Transgenic mice for intersectional targeting of neural sensors and effectors with high specificity and performance. *Neuron* 85, 942–958. [PubMed: 25741722]
- Madisen L, Mao T, Koch H, Zhuo JM, Berenyi A, Fujisawa S, Hsu YW, Garcia AJ, 3rd, Gu X, Zanella S, et al. (2012). A toolbox of Cre-dependent optogenetic transgenic mice for light-induced activation and silencing. *Nature neuroscience* 15, 793–802. [PubMed: 22446880] Development of transgenic ChR2 mice whose expression is tightly regulated, sufficient for optogenetic excitation, and dependent on Cre recombinase for expression.
- Mattis J, Tye KM, Ferenczi EA, Ramakrishnan C, O'Shea DJ, Prakash R, Gunaydin LA, Hyun M, Fenno LE, Gradinaru V, et al. (2012). Principles for applying optogenetic tools derived from direct comparative analysis of microbial opsins. *Nat Methods* 9, 159–172.
- Morgenstern NA, Bourg J, and Petreanu L (2016). Multilaminar networks of cortical neurons integrate common inputs from sensory thalamus. *Nature neuroscience* 19, 1034–1040. [PubMed: 27376765]
- Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, Ollig D, Hegemann P, and Bamberg E (2003). Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. *Proc Natl Acad Sci U S A* 100, 13940–13945. [PubMed: 14615590]
- Petreanu L, Huber D, Sobczyk A, and Svoboda K (2007). Channelrhodopsin-2-assisted circuit mapping of long-range callosal projections. *Nature neuroscience* 10, 663–668. [PubMed: 17435752]
- Petreanu L, Mao T, Sternson SM, and Svoboda K (2009). The subcellular organization of neocortical excitatory connections. *Nature* 457, 1142–1145. [PubMed: 19151697]
- Urban-Ciecko J, and Barth AL (2016). Somatostatin-expressing neurons in cortical networks. *Nat Rev Neurosci* 17, 401–409. [PubMed: 27225074]
- Wall NR, De La Parra M, Callaway EM, and Kreitzer AC (2013). Differential innervation of direct- and indirect-pathway striatal projection neurons. *Neuron* 79, 347–360. [PubMed: 23810541]
- Weiler N, Wood L, Yu J, Solla SA, and Shepherd GM (2008). Top-down laminar organization of the excitatory network in motor cortex. *Nature neuroscience* 11, 360–366. [PubMed: 18246064]
- Wickersham IR, Sullivan HA, and Seung HS (2010). Production of glycoprotein-deleted rabies viruses for monosynaptic tracing and high-level gene expression in neurons. *Nat Protoc* 5, 595–606. [PubMed: 20203674]

- Yizhar O, Fenno LE, Prigge M, Schneider F, Davidson TJ, O'Shea DJ, Sohal VS, Goshen I, Finkelstein J, Paz JT, et al. (2011). Neocortical excitation/inhibition balance in information processing and social dysfunction. *Nature* 477, 171–178. [PubMed: 21796121]
- Zeng H, and Sanes JR (2017). Neuronal cell-type classification: challenges, opportunities and the path forward. *Nat Rev Neurosci* 18, 530–546. [PubMed: 28775344]
- Zhang F, Wang LP, Brauner M, Liewald JF, Kay K, Watzke N, Wood PG, Bamberg E, Nagel G, Gottschalk A, et al. (2007). Multimodal fast optical interrogation of neural circuitry. *Nature* 446, 633–639. [PubMed: 17410168]

Significance Statement

The development of light-based approaches, including channelrhodopsins, to manipulate brain activity has enabled neuroscientists to study the connectivity and function of neural circuits with unprecedented precision. Because different molecules respond to partially overlapping light spectra, using multiple opsins in the same preparation has been a challenge. Efforts to develop new channelrhodopsin variants that respond to different wavelengths have been effective in shifting kinetics and spectral sensitivity. This protocol describes a method to excite two distinct populations with red- and blue-shifted light at different times in order to assess convergence in neural circuits. This method is useful for testing convergence of two inputs in a single neuron.

Figure 1.

Light spectra for excitation of a range of channelrhodopsins. **(A)** Normalized response amplitude for Channelrhodopsin-2 (ChR2 with H134R mutation), Chronos, C1V1, ReaChR, and Chrimson (Boyden et al., 2005; Klapoetke et al., 2014; Lin et al., 2013; Nagel et al., 2003; Yizhar et al., 2011). **(B)** Common LED emission spectra used to excite channelrhodopsins in optogenetic experiments. These are broad or narrow depending on the manufacturer and application. **(C)** Independent multiple channel photostimulation would ideally be performed with idealized opsins whose photosensitivity did not overlap. **(D)** Realistic spectra of current opsins have overlap in their activation spectra. At bottom, selection of stimuli in and out of the response spectra permits activation of red and blue

opsins (blue stimulus) or red opsin alone (red stimulus) during prolonged pulses (top row). Brief blue pulses might be used to separate blue and red opsins based on kinetics and sensitivity (bottom row).

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Figure .2.

Optical arrangements for dual channel photostimulation. **(A)** Use of a filter to block LED illumination below 585 nm was necessary to completely eliminate small, near threshold activation of ChR2. Top, with filter, ChR2 responses to 590 nm LED are eliminated. Bottom, without filter, small responses to 590 nm LED remain. Recordings shown are in separate neurons. **(B)** Arrangement of optical components for photostimulation. Orange (590 nm) and blue (470 nm) LEDs are collimated for homogeneous illumination. These are combined with a dichroic (ET590LP, Chroma). A D607/45 bandpass filter (Chroma, illustrated in red) is used to cut off light from the 590 nm LED below 585 nm. In the microscope body (Olympus BX50WI), a silver mirror (instead of filter cube) is used to reflect maximum light intensity to the specimen plane. Specimen illustrated for clarity (live tissue does not look this good). A 4x/0.16 NA objective was used (other objectives are effective).

Figure 3.

Direct response characterization of a pair of red and blue channelrhodopsins. (A-C) Direct responses to a range of orange (590 nm) and blue (470 nm) LED pulses of ChR2+ (A) and ReaChR+ (B) cortical pyramidal neurons in somatosensory cortex. Maximum photon flux 5.3×10^{15} photons/s * mm² (~2 mW/mm²) matched for orange and blue light. Intensities ranged from 100%, 50%, 25%, 10%, 5%. Pulse duration, 1 s. (C) Normalized peak responses are maximal to 470 nm, and are similar amplitude for responses to 590 nm in ReaChR+ neurons (red), but negligible in ChR2+ neurons (blue). (D-G) Direct responses to a range of orange (590 nm) and blue (470 nm) LED pulses of ChR2+ (D) and ReaChR+ (E) cortical pyramidal neurons in somatosensory cortex. Maximum photon flux 5.3×10^{15} photons/s * mm² (~2 mW/mm²) matched for orange and blue light. Durations ranged from 1, 2, 5, 25, 50 ms. Pulse intensity 100%. (F) Normalized response as a function of stimulus duration for ChR2 (blue) and ReaChR (red). (G) Kinetic differences in time to peak for ChR2 (blue) and ReaChR (red) with differences in stimulus intensity.

Figure 4.

Kinetics of ReaChR activation slow at long wavelengths. **(A)** Direct responses of ReaChR in a cortical pyramidal neuron in somatosensory cortex. Responses are measured for 1, 5, and 50 ms LED pulse duration. Maximum photon flux 5.3×10^{15} photons/s * mm² (~2 mW/mm²) matched for red, orange, and blue light. **(B)** Direct responses, as in **(A)**, but on shorter timescale to emphasize differences in rise kinetics.

Figure 5.

Response of Opsin-positive cortical pyramidal neurons to sustained optical stimulation. (A) Whole-cell recording of L2/3 pyramidal neurons in acute slice of primary somatosensory cortex (S1) expressing ChR2 following viral injection. Left, 300 pA current injection. Right, 600 pA current injection. (B) Brightfield (left) and fluorescence (center) images of S1 expressing ChR2-mVenus following viral injection. The fluorescence image (right) shows the slice image when illuminated with a spot centered over axons in L5 and L6. (C) Top, cartoon of whole-cell recording during axonal stimulation with LED. Bottom, current clamp recording with membrane potential set to -60 mV. 500 ms LED stimulation activates a single action potential (recorded at the soma), followed by sustained depolarization. (D) Mean evoked APs per trial $\pm$ SEM, for recordings with V_m set to -60 mV or -80 mV.

Figure .6.

Persistent current evoked at high expression levels. **(A)** Left, cartoon of the synaptic recording configuration. Right, normalized postsynaptic currents evoked during 1 s photostimulus. Top recording shows three different experiments with ReaChR in 3 mice, injected with ~60 nL of AAV2/1-Syn-ReaChR-mCitrine (~2E13 GC/ml titer) at various dilutions. Black, 1/50 dilution; dark gray, 1/9 dilution; light gray, 1/1 dilution. Bottom recording shows the same experiment with ChR2 in 1 mouse, injected with ~60 nL of AAV2/1-CAG-ChR2-tdTomato (~1E13 GC/ml titer). N indicated on plot. **(B)** Quantification of persistent current amplitude. **(C)** TTX treatment did not abolish the persistent current from pyramidal neurons. Example recording compares persistent current before (black) and after (red) application of 1 μ M TTX. Magnitude of persistent current remaining in TTX plotted at right (mean $\pm$ SEM, N=3). **(D)** Delay to onset of persistent current, as measured in 1 μ M TTX. Left, example recording before (black) and after (red) TTX application. Right, quantification of mean onset time in ms.

Figure .7.

Dual channel photostimulation is effective in certain axon populations and not in others. (**A-C**) Corticostriatal inputs from primary sensory (S1, expressing ChR2) and primary motor (M1, expressing ReaChR) cortex project to medium spiny neurons (MSNs) in the striatum. These inputs can be excited sequentially as described above. (**D-F**) Excitation of somatostatin+ interneurons expressing ChR2 (SOM-ires-Cre x Ai32) results in an initial IPSC in pyramidal neurons, followed by a substantial continuous inhibitory current.

Figure 8.

Synaptic responses from dual channel photostimulation. **(A)** Cartoon illustrating whole-cell recording from a L2/3 neuron in primary motor cortex (M1) while stimulating ReaChR+ axons from primary somatosensory cortex (S1) and thalamus (PO). **(B)** LED stimulation with 590 nm and 470 nm light. LED stimulation paradigms are as described in Step 6 of Basic Protocol 1. Interstimulus intervals from 590 nm LED onset to 470 nm LED onset are 50 ms, 100 ms, and 250 ms. Stimulation intensity $\sim 2 \text{ mW/mm}^2$ for each color, matched for photon flux. Right traces are the same as left traces, with 500 ms 590 nm stimulation (orange only) subtracted to reveal only ChR2-evoked synaptic responses. **(C)** Amplitude of ChR2 responses, normalized to the longest ISI (250 ms) as a function of delay time prior to activation of 470 nm LED. Gray, mean responses $\pm$ SEM. N=37 neurons. **(D)** Onset delay in ms from LED illumination (590 nm – ReaChR; 470 nm – ChR2) until synaptic response reaches 10% of peak value. **(E)** Rise time of EPSC from 10% to 90% of peak value. **(F, G)** Brightfield (top) and fluorescent (bottom) images of coronal section of M1. ReaChR-mCitrine (green) and ChR2-tdTomato (red) axons overlap near the recording site (pipette in brightfield image).

Figure 9.

Comparison of current amplitude measured sequentially instead of via subtraction. (A) Cartoon of stimulation paradigm with presynaptic afferents from thalamus (PO, expressing ChR2) and cortex (somatosensory cortex, expressing ReaChR) exciting pyramidal neurons in primary motor cortex. (B) Example trace from LED stimulation paradigms including blue LED alone (Combined, illustrated in black), orange LED alone (ReaChR, illustrated in orange), and orange followed by blue LED after 250 ms to isolate ChR2-mediated response alone (ChR2, illustrated in blue; the trace shown is the subtraction of raw trace from orange only). (C) Schematic of how current amplitudes were measured for orange (o), blue (b), and combined (c) currents. (D) Ratio of combined versus sum of the individual currents (N=27).