eNeuro

Research Article: Methods/New Tools | Novel Tools and Methods

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https://doi.org/10.1523/ENEURO.0378-22.2023

Cite as: eNeuro 2023; 10.1523/ENEURO.0378-22.2023

Received: 12 September 2022
Revised: 21 February 2023
Accepted: 23 February 2023

Manuscript title:

Bicistronic expression of a high-performance calcium indicator and opsin for all-optical stimulation and imaging at cellular resolution

Abbreviated title:

A bicistronic construct for all-optical stimulation

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PKL, BA, and MH designed the construct. ZZ performed viral injections and surgical implants. AL developed injection and dual-virus strategies. PKL collected two-photon data. ZZ collected widefield fluorescence data. PKL analyzed the data. YD, AL, and MH designed and constructed the two-photon microscope and two-photon holographic stimulation equipment. PKL, NGF, and MH wrote the manuscript.

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Number of figures: 4

Number of extended figures: 5

Number of words: Abstract (162), Significance statement (94), Introduction (789), Discussion (1204)

Acknowledgments:

We thank Georg Jaindl and others from Vidrio/MBF Biosciences for expert assistance and support, Victoria M. Scott for assistance in virus testing, and Tommaso Fellin for comments on the manuscript. This work was supported by the NIH BRAIN Initiative, grant U19NS107464, and the NIMH Intramural Research Program, ZIAMH0020967.

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Abstract

State-of-the-art all-optical systems promise unprecedented access to neural activity *in vivo*, using multiphoton optogenetics to allow simultaneous imaging and control of activity in selected neurons at cellular resolution. However, to achieve wide use of all-optical stimulation and imaging, simple strategies are needed to robustly and stably express opsins and indicators in the same cells. Here we describe a bicistronic adeno-associated virus (AAV) that expresses both the fast and bright calcium indicator jGCaMP8s, and a soma-targeted (st) and two-photon-activatable opsin, ChrimsonR. With this method, stChrimsonR stimulation with two-photon holography in the visual cortex of mice drives robust spiking in targeted cells, and neural responses to visual sensory stimuli and spontaneous activity are strong and stable. Cells expressing this bicistronic construct show responses to both photostimulation and visual stimulation that are similar to responses measured from cells expressing the same opsin and indicator via separate viruses. This approach is a simple and robust way to prepare neurons *in vivo* for two-photon holography and imaging.

Significance statement

New multiphoton photostimulation methods, combined with standard two-photon calcium imaging, can yield unprecedented levels of control for dissecting brain circuit function *in vivo*. These all-optical methods rely on an interplay between optogenetics and calcium indicators, to both measure and control neural activity. However, genetic strategies to achieve reliable and stable co-expression of opsin and indicator are often challenging to execute. Here, we present a genetic tool to achieve robust co-

expression of jGCaMP8s indicator and stChrimsonR opsin via a single injected virus. This approach facilitates all-optical experiments to investigate the circuit principles underlying brain activity.

Introduction

Perception and action depend on neural computations, created as patterns of activity propagate in neural circuits. Means to monitor and control these activity patterns are important tools to study how brain function governs perception and behavior. Optogenetics is a valuable approach for controlling genetically-specified sets of neurons. However, achieving optical specificity — the ability to select a single cell and perturb it — is challenging. Conventional one-photon excitation is not ideal for single cell stimulation in tissue, as one-photon excitation can lead to undesired activation above and below the targeted focal plane (Denk et al., 1990). Moreover, even in the focal plane, one-photon methods can be limited in their ability to restrict excitation to small volumes due to light scattering in the tissue (Denk et al., 1994), and this constraint becomes more severe with depth in the tissue.

Two-photon optogenetics overcomes these limitations, enabling perturbations in selected single cells (Rickgauer and Tank, 2009; Packer et al., 2012, 2015; Emiliani et al., 2015; Adesnik and Abdeladim, 2021). With this approach, using a stimulation laser and an imaging laser independently focused at different locations deep in the brain, it is possible to measure evoked activity patterns as stimulation is delivered. Two-photon optogenetics has been used to study within-area network dynamics (Chettih and Harvey, 2019), and to understand how chosen patterns of activity evoked by stimulation influence perception (Carrillo-Reid et al., 2019; Marshel et al., 2019; Dalgleish et al., 2020; Gill et al., 2020; Robinson et al., 2020; Daie et al., 2021; Rowland et al., 2021; Russell et al., 2022).

A variety of opsins and calcium indicators have been used for two-photon stimulation and simultaneous imaging (Shemesh et al., 2017; Mardinly et al., 2018; Chen et al., 2019; Marshel et al., 2019; Adesnik and Abdeladim, 2021; Forli et al., 2021; Sridharan et al., 2022). Desirable properties for calcium indicators used with two-photon stimulation include high sensitivity, to measure small changes in neural

firing, and fast dynamics, to monitor quickly-changing spike trains. Desirable properties for opsins include fast dynamics to allow precise control of spiking, and the ability to be activated using moderate to low stimulation intensities. This allows many neurons to be stimulated with low total energy levels delivered to the brain.

However, expressing both an opsin and a calcium indicator in the same cells has proven challenging, especially to achieve stable expression levels of both proteins for weeks to months. High levels of indicator expression in single cells can lead to reduced fluorescence responses, typically with constant levels of bright fluorescence. This phenomenon of bright, non-responsive neurons with high levels of calcium indicator expression can become more common as time elapses after transfection (Tian et al., 2009; Chen et al., 2013; Packer et al. 2015). Co-expression of the two proteins with two individual viruses allows fine-tuning and optimizing the expression level of each protein separately, but with this approach it can be challenging to achieve co-expression in many neurons (see Packer et al., 2015; Carrillo-Reid et al. 2018, their Fig. 4a; Chettih and Harvey, 2019; Gill et al., 2020; Russell et al., 2022). Genetic mouse lines promise to simplify this co-expression process (Bounds et al., 2022), but current genetic lines have limited combinations of opsin and calcium indicator available, and in genetic lines it has not always been possible to achieve the levels of indicator expression (Daigle et al., 2018) that give imaging quality comparable to viral expression.

To address these issues, here we demonstrate a single Cre-dependent virus that expresses both opsin and indicator in transfected cells without requiring multiple overlapping viral injections. Our solution uses the ChrimsonR opsin, targeted to cells' somata with a Kv2.1 domain (soma-targeted ChrimsonR, stChrimsonR, Pégard et al., 2017), and jGCaMP8s, a bright, sensitive genetically encoded calcium indicator (Zhang et al., 2021). The genes are linked by the self-cleaving peptide P2A (Szymczak et al., 2004; Prakash et al., 2012), an approach previously used with GCaMP6m and the opsin ChRmine (Marshel et al. 2019). stChrimsonR is an opsin with fast on- and off- kinetics (Klapoetke et al., 2014) with a red-shifted excitation spectrum (Sridharan et al., 2022) and moderate sensitivity to two-photon

activation (Mardinly et al., 2018; Chen et al., 2019). This moderate sensitivity to stimulation and red-shifted excitation spectrum gives the advantage of allowing neurons to be driven by the stimulation laser, while reducing potential activation, or crosstalk, from the (lower peak intensity and blue-shifted) imaging laser. jGCaMP8s is from the latest generation of fast calcium indicators, balancing needs for a bright signal and physiologically relevant kinetics. We find this construct provides a stable preparation for long-term experiments with repeated stimulation. With this single virus strategy, we achieve widespread and stable expression, effective and precise holographic stimulation of many cells, and high-quality recording of neural activity.

Materials and methods

Virus

Both pAAV-hSyn-DIO-ChrimsonR-mRuby2-ST (Addgene Plasmid #105448, RRID:Addgene_105448) and pGP-AAV-syn-jGCaMP8s-WPRE (Addgene Plasmid #162374, RRID:Addgene_162374) plasmids were used to build the pAAV-hSyn-DIO-jGCaMP8s-P2A-stChrimsonR construct and also used to make viruses expressing each protein individually. The mRuby fluorescent tag from pAAV-hSyn-DIO-ChrimsonR-mRuby2-ST was removed, and the sequence encoding jGCaMP8s was cloned into the construct along with a P2A peptide linker. The plasmid was used for packaging into an adeno-associated virus (AAV9). The plasmid version of this construct will be available on Addgene (Plasmid #174007, RRID:Addgene_174007).

Animals and surgery

All animal procedures were performed in accordance with the NIH Institutional Animal Care and Use Committee's (IACUC) regulations. *Emx1-Cre* mice (The Jackson Laboratory; RRID:IMSR_JAX:005628) were used in all experiments to target expression of Cre to excitatory, glutamatergic neurons. N = 8 total animals were used in this study (N = 4 male, N = 4 female); no differences due to sex were noted in the results. Mice 2 months of age or older were anesthetized with isoflurane (1–3% in 100% O_2 at 1 L/min) and kept on a heating pad for warmth. An intraperitoneal injection of dexamethasone (3.2 mg/kg)

was administered before incision to reduce inflammation. The skull was exposed, and a custom metal head post was positioned at the base of the skull. A 3mm diameter circular craniotomy was made over the left hemisphere of primary visual cortex (ML -3.1 mm, AP +1.5 mm relative to Lambda) using an air driven dental drill (Aseptico; Woodinville, WA) with a Neoburr drill bit (Friction Grip 1/4; Microcopy; Kennesaw, GA). AAV9-hSyn-DIO-jGCaMP8s-P2A-stChrimsonR was diluted in phosphate-buffered saline (PBS), and 2 nmol sulforhodamine 101 was added to visualize injection progression in the brain. We tested a small range of viral titers (2.6-4.7x10^{12} GC/mL) in 6 animals and found all these titers allowed for reliable data collection. (Titers: 2.6x10^{12} GC/mL in mouse 1, 3, 4, and 6; 3.4x10^{12} in mouse 2; 4.7x10^{12} in mouse 6.)

For 'dual-virus' injected animals, we instead diluted in PBS two separate viruses: AAV9-hSyn-jGCaMP8s-WPRE (final titer 1.0x10^{13} GC/mL) and AAV9-hSyn-DIO-stChrimsonR-mRuby2 (final titer 2.7x10^{12} GC/mL).

Viruses were injected unilaterally with a stereotactic syringe pump (Stoelting; Wood Dale, IL) through a pulled glass pipette tip cut to an opening of 10–15 µm diameter. Injections were targeted to 200 µm below the surface of the brain and administered at a rate of 0.1 µL/min for a total volume of 300 nL per injection site (5–10 injection sites). A 3 mm optical window (Tower Optical; Boynton Beach, FL) was implanted over the craniotomy. Both the optical window and metal head post were fixed to the skull using C&B metabond dental cement dyed black (Parkell; Edgewood, NY). Last, a custom-made removable light-blocking cover was fixed atop the implant to prevent ambient light exposure to the opsin. Animals were individually housed after surgery. Mice were imaged three or more weeks post-injection. All animals were housed in a 12:12 hour reverse light-dark cycle and allowed food and water ad libitum.

Widefield fluorescence imaging

Widefield fluorescence imaging was done using a Discovery stereo microscope (Zeiss; Jena, Germany) with an X-Cite XYLIS LED source (Excelitas; Mississauga, Canada) and a blue excitation and green

emission filter set (KSC XXX-814; Kramer Scientific; Amesbury, MA). Images were collected (200 ms exposure period) using a Retiga R3 CCD camera (QImaging, Inc.; Surrey, BC).

In vivo two-photon calcium imaging

To perform two-photon calcium imaging, animals were first head-fixed under a 16x water-immersion objective (Nikon; Tokyo, Japan). Imaging was performed using a custom-built microscope using MIMMS (Modular In vivo Multiphoton Microscopy System) components (Sutter Instruments; Novato, CA) and a Chameleon Discovery NX tunable femtosecond laser (Coherent, Inc.; Santa Clara, CA). Imaging was controlled using ScanImage software (MBF Biosciences; Williston, VT) in MATLAB. A small volume (~1 mL) of clear ultrasound gel was placed over the optical window to immerse the objective. Calcium responses were measured at approximately 100–200 μm below the surface of the pia in layers 2/3 of primary visual cortex using a 414 x 414 μm field of view, except where noted. Imaging was performed via bidirectional raster scanning with a resonant-galvo system (8 kHz resonant scanner, 512 lines, ~30 Hz frame rate) using 920 nm wavelength light at 15–20 mW measured at the front aperture of the objective (pulse rate 80 MHz, pulse energy 0.19–0.25 nJ/pulse).

In vivo two-photon holographic photostimulation

Holographic photostimulation was performed using a Satsuma femtosecond pulsed laser (Amplitude Laser; Pessac, France) at 1030 nm wavelength along a second optical path (galvo-galvo) integrated into the two-photon microscope just before the tube lens using a polarizing beam combiner. A spatial light modulator, or SLM (1920 x 1152 pixels; Meadowlark Optics, Frederick, CO), was used to generate holographic patterns of 10 μm diameter disks within a 2-dimensional plane (aligned to the imaging focal plane). The SLM was followed by a relay lens system (two achromatic lenses, with focal lengths 250 mm and 100 mm) in a 4-f configuration between SLM and galvanometers. Galvonometers were used to direct the diffracted light pattern from the SLM onto the sample, pointing the center of the diffracted pattern to the center of the targeted cells to maximize diffraction efficiency. The zero order (undiffracted beam) from the SLM was blocked using a small amount of furnace cement (30–40 μm diameter) on a

196 glass slide. Stimulation targets were defined and SLM phase masks were computed using ScanImage
197 software (MBF Biosciences; Williston, VT). The radial point-spread function (PSF) of diffraction limited
198 spots generated by the SLM was 0.73 μm and the axial PSF was 9.85 μm.
199
200 Stimulation was applied for intervals of either 150 ms at 2 mW/target (Fig. 4A-H) or 300 ms at 2.5
201 mW/target (Fig. 4I-L) with a 500 kHz pulse rate (pulse energy 13 or 16 nJ/pulse). The laser was gated
202 on when the imaging resonant galvo was reversing direction during bidirectional scanning, and off
203 during the imaging pixel acquisition (on time 19 μs, off time 44 μs, duty cycle 30%). This fast gating
204 allows for imaging neural responses during stimulation periods, by collecting imaging data on each line
205 while stimulating in between lines, switching at rates (period 62.5μs) much faster than the onset
206 kinetics of the opsin (>1 ms; Klapoetke et al., 2014; Mardinly et al., 2018; Sridharan et al., 2022). To
207 perform the fast gating, the imaging frame clock was inverted via a TTL logic gate; Pulse Research
208 Lab; Torrance, CA; see Mardinly et al. (2018) for example circuit. We report average stimulation power
209 over the milliseconds-long stimulation pulses: that is, the average power we report is found by
210 multiplying the peak stimulation power while the laser is on during the fast pulses by the duty cycle of
211 the fast pulses. For example, for Fig. 4A-H, the average power per target was 2 mW (the number we
212 report), while the peak power of the laser over the target was 6.5 mW and the duty cycle 30%.
213
214 Retinotopic mapping
215 To map the retinotopic position of visual stimuli in V1 under the optical window, we performed
216 hemodynamic intrinsic imaging in awake head-fixed animals. We presented visual stimuli (drifting
217 square wave gratings, 0.1 cycles/degree, 10 degrees diameter) for 5 seconds (with 10 seconds
218 between presentations) at different retinotopic positions and measured reflected 530 nm light on the
219 brain to quantify hemodynamic-related changes in absorption. The 530 nm light was delivered using a
220 fiber-coupled LED (M530F2; Thorlabs, Newton, NJ) and imaging was collected on the same stereo
221 microscope used for widefield fluorescence imaging using a 1x widefield objective and a green long-
222 pass emission filter. Imaging was acquired at 2 Hz. Changes in reflectance were computed for every

stimulus location between a baseline period (5 seconds prior to stimulus onset) and a response period (2.5 second window starting 3 seconds after stimulus onset). A centroid of the hemodynamic response was computed for each stimulus location and an average retinotopic map was fit to the positions of the centroids. Retinotopic maps were then used to guide stimulus locations for two-photon imaging measures of visual responses to Gabor stimuli.

Visual stimulation

To measure visual responses during two-photon imaging, we presented awake animals with Gabor patches (sinusoidal drifting gratings filtered with a gaussian mask with 15 degree full-width half-max) with spatial frequency 0.1 cycles/degree for 2 second periods (6 seconds of gray screen between presentations) at 100% contrast. An LCD monitor with neutral gray background was used to present visual stimuli and was positioned approximately 20 centimeters in front of the animal. Drifting gratings of 8 different directions (45 degree increments) were presented in random order across trials. Each direction was presented for 20 repetitions.

Two-photon calcium imaging analysis

Two-photon calcium imaging data was first downsampled from 512 x 512 pixels to 256 x 256 to ease handling of data. Background correction was performed by computing the average intensity image across frames and subtracting the minimum pixel value of this average from the image stack. All remaining negative pixel values (due to noise) were then set to zero. We motion corrected all images using the CaImAn toolbox (Giovannucci et al., 2019) and performed cell segmentation using Suite2p to allow manual selection of cell masks (Pachitariu et al., 2017). Fluorescence intensity traces were then calculated as the average intensity across all pixels within a cell's segmented mask. To quantify cell activity, we computed $\Delta F/F_0$ for each cell. F_0 was defined as the average fluorescence across the 50 imaging frames that occurred directly prior to stimulus presentation for all trials (N = 160 trials, Fig. 2, 3; N = 50 trials, Fig. 4C; N = 100 trials, Fig. 4K) in an imaging session.

Reliability measures for visual responses were quantified via 2-sample t-test (with Bonferroni correction for multiple comparisons within each experiment) using $\Delta F/F_0$ values between the visual stimulation period (N = 60 frames) and an equivalent number of frames preceding stimulus onset across all trials. Photostimulation responses were determined by averaging $\Delta F/F_0$ activity over a 1 second period (N = 30 frames) following stimulus onset and assessed using a 7.5% $\Delta F/F_0$ threshold (Extended Fig. 4-2).

For visual display of $\Delta F/F_0$ responses across the field of view (FOV; in Fig. 2B, Fig. 3B, Fig. 4B and Extended Fig. 4-3B), F_0 was computed for every pixel in the same manner as for cell-based calculations. We then smoothed the F_0 image using a gaussian filter ($\boldsymbol{\sigma}$ = 20, radius = 80.5 pixels) to act as a means of local contrast adaptation. F was computed at every pixel as the average value across a response window (N = 60 frames, Fig. 2B and Fig. 3B; N = 10 frames, Fig. 4B and Extended Fig. 4-2B; N = 15 frames, Fig. 4) following stimulation onset and across all trials (for Fig. 2B and Fig. 3B, N = 20 trials/stimulus direction).

To assess the lateral extent of photostimulation, we quantified the average activity across 40 x 40 μm regions centered on targeted cells (from Fig. 4B). To control for activation of surrounding neuropil across the entire field of view, we subtracted an average neuropil ROI centered on 50 random coordinates in the field of view (Fig. 4F-G). A Gaussian specified by:

$$y = a * e^{-(x-b)^2/2c^2}$$

was fit to the one-dimensional cross-section of the average cell-centered response ROI (Fig. 4G). The lateral extent of a photostimulation disk target (10 μm) was found as the full width at half-max of this Gaussian fit.

Half-decay time of photostimulation responses

To assess the decay time of photostimulation responses, stimulus-triggered average responses were computed for each cell. Exponential decay functions of the form:

$$y = a * e^{-\tau x}$$

were fitted to the 600 ms (20 frames at 30 Hz) period immediately after photostimulation offset. Using the estimated time constant, τ, the half-decay time was calculated as:

half-decay time = ln(2) / τ.

Visual tuning analysis

To quantify visual tuning in individual cells, we computed a direction selectivity index (DSI), orientation selectivity index (OSI), and global orientation selectivity index (gOSI) for all visually-responsive cells following the methods of Kondo and Ohki, 2016. For each metric, 0 indicated no selectivity and 1 indicated maximal selectivity. We first calculated a tuning curve for each cell as the average $\Delta F/F_0$ activity across the entire visual stimulus period (N = 60 frames, or 2 seconds) and across all trials for each of the 8 stimulus directions (N = 20 trials/direction). Responsivity was calculated as the average value across the 8 directions in the tuning curve. The stimulus direction corresponding to the peak value of this tuning curve was determined to be the preferred direction for a cell. The response at this direction, $R_{prefDir}$, as well as the opposite direction (180 degrees away), $R_{oppoDir}$, was used to calculate the DSI as:

$$DSI = (R_{prefDir} - R_{oppoDir}) / (R_{prefDir} + R_{oppoDir}).$$

To calculate OSI, we first averaged opposite pairs of directions of the tuning curve to yield average responses to each of the 4 stimulus orientations. The preferred orientation of cells was determined to

be the peak value between the 4 orientations. The response at the preferred orientation, $R_{prefOri}$, as well as the response at the orthogonal orientation (90 degrees away), $R_{orthOri}$, was used to calculate the OSI as:

$$OSI = (R_{prefOri} - R_{orthOri}) / (R_{prefOri} + R_{orthOri}).$$

Last, the gOSI for each cell was computed via a vector averaging method (Swindale, 1998):

$$gOSI = sqrt((\sum R_i * \sin 2\theta_i^2) + (\sum R_i * \cos 2\theta_i^2)) / \sum R_i,$$

where *i* represents the *i*th direction (equivalent to 1 - circular variance).

Results

We made a single adeno-associated virus (AAV; Fig. 1A) that contains the genes for jGCaMP8s (Zhang et al., 2021) and ChrimsonR (Klapoetke et al., 2014), separated by the P2A cleavage site. This bicistronic expression strategy allows for the reliable co-expression of both proteins in different cells (Tang et al., 2009). The Cre dependence is provided by the DIO (FLEX) strategy (Schnütgen et al., 2003; Cardin et al., 2009), such that without recombination, the genes are in the antisense orientation to limit leaky expression in the absence of Cre.

Robust expression of jGCaMP8s and stChrimsonR using a bicistronic construct

For all experiments, we injected the virus into primary visual cortex (V1) of adult *Emx1-Cre* mice, to yield expression in excitatory neurons (Fig. 1B). We implanted optical windows over V1 for imaging.

At three weeks post-injection, strong GCaMP fluorescence was visible using fluorescence imaging of the cortical surface through the optical window (N = 6 animals injected and measured 22-53 days post-injection; Fig. 1C and Extended Fig. 1-1). To assess expression in individual cells, we used *in vivo* two-photon calcium imaging. We found robust expression in many cells across multiple imaging depths (Fig. 1D). To assess crosstalk due to imaging, where the imaging laser might in principle activate the opsin, we measured average cell activity and found no sign of crosstalk (2/12 imaging sessions showed significant increases over the first two seconds of imaging, $p<0.05$; 4/12 showed significant decreases, $p<0.05$; Mann-Whitney U-test of cell fluorescence between 1st second and 2nd second, Bonferroni correction for multiple comparisons; see Extended Fig. 1-2 for timecourses).

We find that many or all neurons show GCaMP fluorescence throughout the cell, unlike what is seen with expression of GCaMP alone under control of a single promoter, where GCaMP is often excluded from the nucleus (Tian et al., 2009; Chen et al., 2013; Packer et al. 2015). Because this might imply some differences in GCaMP trafficking for this bicistronic virus compared to GCaMP expressed with a single promoter, we next compare responses of neurons transfected with this bicistronic virus versus those from neurons transfected with two single viruses that express each of the two proteins (jGCaMP8s and stChrimsonR) separately. The pattern of responses for the two approaches is very similar, suggesting that even if there are some trafficking differences, our bicistronic GCaMP is functioning normally.

In vivo recording of visually-evoked and spontaneous activity of V1 cells expressing jGCaMP8s-P2A-stChrimsonR

In order to determine whether cells expressing jGCaMP8s-P2A-stChrimsonR exhibit physiologically expected sensory-evoked responses (see Kondo and Ohki, 2016; Zhang et al., 2021; Bounds et al., 2022), we used two-photon imaging to record jGCaMP8s activity from excitatory cells in layer 2/3 of mouse V1 during and between presentations of drifting grating (Gabor patches, 15 degree full-width half-max, FWHM) stimuli. We presented gratings across eight orientations (N = 20 repetitions per

orientation) in random order and computed trial-average $\Delta F/F_0$ for different orientations. We found strong and widespread visually-evoked activity across the field of view (Fig. 2A-B). To assess visual selectivity and responsiveness of cells, we calculated single-cell $\Delta F/F_0$ activity traces across visual presentations and selectivity indices for grating direction (DSI) and orientation (OSI and global OSI, or gOSI) (Fig. 2C shows selectivity in 4 example cells and Fig. 2D displays activity in 20 example cells across three consecutive trials from one animal). We found many cells respond to drifting grating stimuli (913/1046 cells responsive across N = 3 animals, 2-sample t-test, stimulus vs. baseline, all stimulus directions pooled; $p<0.001$ threshold; test done with Bonferroni correction across neurons within each experiment). Many neurons also exhibit spontaneous activity (Fig. 2D) between grating presentations.

We first compared visual responses to previous reports from other laboratories. We examined tuning for the direction and orientation of the stimuli and found the distribution of neurons' tuning was very similar to what has been previously reported with GCaMP6 or GCaMP7 (Kondo and Ohki, 2016; Bounds et al., 2021; and see Neil and Stryker, 2008 for a comparison to electrode recordings). For every cell, in addition to selectivity indices, we also measured preferred direction and orientation and overall responsivity (average activity across tuning curve, see Methods for details; Fig. 2G, green curves). We found the distributions of these tuning metrics align closely with prior reports using calcium indicators (GCaMP6s, Kondo and Ohki, 2016, see Fig. 3D, Fig. 4D, supp Fig. 7D; GCaMP7s, Bounds et al., 2022, see Fig. 3D). We find that preferred orientations and directions are evenly distributed (Fig. 2G) and tuning index distributions are similar to what has been previously reported. In these reports, mean DSI was 0.27 (Kondo and Ohki, 2016), while our mean DSI was 0.21 ± 0.21 (1 std. dev.) Similarly, their mean OSI was 0.62 (Kondo and Ohki, 2016) and 0.56 (Ai203; Bounds et al., 2022), while we report a mean OSI of 0.46 ± 0.28. For gOSI, they report a mean of 0.46 (Kondo and Ohki, 2016), while we report a mean gOSI of 0.31 ± 0.18.

Next, we examined how the bicistronic construct's visual responses compare to visual responses in data we obtained from transfection with two different viruses, each carrying jGCaMP8s or stChrimsonR

(560/643 cells responsive across N = 2 animals, 2-sample t-test, stimulus vs. baseline, all stimulus directions pooled; $p<0.001$ threshold; test done with Bonferroni correction across neurons within each experiment). Across all measures of selectivity, stimulus preference, and responsivity, we found that distributions of cell metrics align closely (Fig. 2E). These data demonstrate that expression of jGCaMP8s-P2A-stChrimsonR allows for reliable two-photon measurements of sensory-evoked and ongoing calcium activity.

Stability of visual responses over time in V1 cells expressing jGCaMP8s-P2A-stChrimsonR

A challenging aspect of long-term all-optical experiments is maintaining satisfactory levels of expression over weeks to months. To assess the suitability of our construct for longer-term experiments, we next measured sensory-evoked activity in the same FOV of multiple animals a month apart. Again, we found that V1 cells expressing jGCaMP8s-P2A-stChrimsonR produce robust visually-evoked responses to drifting grating stimuli (Fig. 3A-B). Using tuning selectivity metrics calculated from responses to gratings of 8 different directions (DSI, OSI, and gOSI), as well as a measure of overall responsivity (average across tuning curve activity, see Methods for details), we found no substantial change in cell responses from two animals a month apart in the same FOV (Fig. 3C-D). These results indicate that measures of activity from cells expressing our construct are reliably maintained over the span of several weeks, enabling longer-term experiments.

Holographic pattern stimulation in vivo of cells expressing jGCaMP8s and stChrimsonR

One goal of achieving co-expression of an indicator and an opsin is to facilitate all-optical interrogation of brain circuits. Therefore, to test the ability of cells expressing the construct to respond to optogenetic stimulation, we measured jGCaMP8s activity via two-photon imaging *in vivo* (920 nm imaging wavelength, 15–20 mW imaging power, 80 MHz pulse rate, 0.19–0.25 nJ/pulse) while stimulating cells with holographic light patterns (1030 nm, 500 kHz pulse rate, 13–16 nJ/pulse). We used holography to simultaneously stimulate cells in mouse V1 with 10 µm-diameter light spots in a single depth plane.

This stimulation (20 targets, 2.0 mW/target for 300 ms, see Methods for details, Fig. 4A) led to clear stimulation-evoked responses in targeted cells (Fig. 4B-D).

We compared the timecourse of stimulation responses produced with this bicistronic virus to those from neurons transfected with stChrimsonR and jGCaMP8s using two different viruses. We found decay times were similar for the two methods (Extended Fig. 4-1), suggesting jGCaMP8s was functioning similarly for both viral expression strategies. In fact, the variability in decay times was smaller with this bicistronic virus (Extended Fig. 4-1C), perhaps because of lower expression variability of the two proteins with this virus compared to a two-virus approach.

We observed reliable responses to stimulation in a large majority of targeted cells and a few nearby, unstimulated cells (Fig. 4A-D, 19/20 targeted cells and 5/69 unstimulated cells above a 7.5% $\Delta F/F_0$ threshold, see Extended Fig. 4-2 for full distribution of responses, Methods). These reliable stimulation responses were seen consistently across animals (N = 3 mice, N = 10 or 12 targeted cells, 30/32 total targeted cells and 35/1037 unstimulated cells show responses above a 7.5% $\Delta F/F_0$ threshold; Extended Fig. 4-3, Extended Fig. 4-4).

To understand the extent to which the stimulation pattern was precisely exciting the targeted cells and not adjacent cells, we computed the average response around each targeted cell (Fig. 4E). We found that the 10 μm excitation light disks we used produced very localized activation (full width at half-max, 13.5 μm estimated via Gaussian fit, Fig. 4H). Given we observed responses in a fraction of nearby unstimulated cells, we further compared the photostimulation response in unstimulated cells as a function of distance to the nearest stimulated cell. We find that only cells nearby to stimulated cells (within <40 μm) show a positive response to stimulation, with no responses seen at greater distances (Extended Fig. 4-4). These results are in agreement with evidence that cell-specific stimulation drives localized activity in nearby cells within a distance of <40 μm (Packer et al., 2015; Oldenburg et al., 2022).

The holographic approach allows stimulation of many cells simultaneously, and we checked the effects of stimulation at larger numbers of neurons. This stimulation (50 targets, 2.5 mW/target for 150 ms, Fig. 4I-L) also produced consistent activation. We found 33/50 targeted cells showed reliable activation (7.5% $\Delta F/F_0$ threshold, although some cells are activated more strongly than others, Fig. 4L and Extended Fig. 4-3). When giving input to larger numbers of neurons (e.g., 50), it might be expected that response reliability would be lower than when driving smaller numbers of neurons (e.g., 20, Fig. 4A-D), due to recurrent network effects when some stimulated neurons affect other nearby cells (Marshel et al., 2019; Dalgleish et al., 2020; O'Rawe et al., 2022; Oldenburg et al., 2022). We also find more off-target activation in nearby cells when using 50 stimulation targets (45/298 unstimulated cells above a 7.5% $\Delta F/F_0$ threshold, Extended Fig. 4-2 for full distributions, Extended Fig. 4-4 for distance-dependent effects), when compared to 20 stimulation targets (Fig. 4C). It seems likely that this increase in off-target activation is also due to network effects via postsynaptic summation. In sum, we find that a majority of jGCaMP8s-expressing cells selected for holographic targeting show consistent responses to repeated photostimulation, indicating these cells are successfully co-expressing both indicator and opsin.

Discussion

In order to facilitate two-photon holographic photostimulation experiments with simultaneous calcium imaging, we designed a Cre-based, single viral approach to express both opsin and indicator in Cre-expressing cells of interest. The single viral approach simplifies experimental preparation by reducing variation due to relative concentrations of opsin and indicator. Our results indicate that cells expressing AAV9-hSyn-DIO-jGCaMP8s-P2A-stChrimsonR exhibit strong and consistent responses to targeted holographic photostimulation. Also, visual responses measured using this approach have population distributions similar to those previously reported with expression of calcium sensors, indicating normal visual function is maintained and neural activity can be reliably recorded.

Robust activation of neurons with ChrimsonR likely reflects changes in firing rates, instead of precisely timed single spikes

A central appeal of two-photon holography is the ability to dynamically stimulate different groups of cells based on functional properties. However, a limiting factor for such experiments is achieving reliable co-expression of opsin and indicator in many cells throughout the tissue. Recent studies using a variety of opsins and genetic approaches typically find many, but not all cells are responsive to photostimulation (for example, ~80% with Ai203 transgenic line, Bounds et al., 2022; ~90% with ChRmine, ~85% with ChroME2s, ~75% ChroME2f, Sridharan et al., 2022). On par with prior reports for other opsins, we found similar or greater levels of photoactivatable cells in our experiments (~80% of cells photoactivatable; Fig. 4, Extended Fig. 4-2, Extended Fig. 4-3, Extended Fig. 4-4). However, our data differ from previous reports with ChrimsonR, which suggest very few ChrimsonR cells are reliably activated with two-photon stimulation (~10%, see Fig. 7C-D of Sridharan et al., 2022). This report differs from ours in that they aimed to induce precisely-timed single spikes, and thus used much shorter stimulation periods (5 ms, repeated 5 times at 30 Hz). Also supporting the idea that stChrimsonR can be used to reliably activate cells, Mardinly et al. (2018) found roughly 60% of stChrimsonR-expressing neurons were reliably photoactivated (5 ms stimulation at 0.4 mW/μm^2, 1040 nm, 2 MHz pulse rate; however, pulse rate differences with our 500 kHz make direct power comparisons difficult). Thus, while ChrimsonR activation produces relatively smaller photocurrents than the ChroME and ChRmine opsins, our work, as well as past work, suggests that stChrimsonR can indeed be used to reliably activate cells *in vivo*.

It could be possible, in fact, that smaller stimulation currents, which modulate the firing rate of neurons without reliably driving single spikes with precise timing, are similar to inputs neurons receive during many forms of visual sensory stimulation. During flashed or drifting grating stimuli (Niell and Stryker, 2008; Busse et al., 2011; Glickfeld et al., 2013), mouse neurons change their firing rates by only up to tens of spikes per second or less, and fire with irregular, Poisson-like timing. Electrophysiological experiments show that activation of stChrimsonR in the visual cortex with longer pulses produces an

484 elevation of neurons' firing rates while neurons' firing remains irregular, as occurs with sensory stimuli
485 (O'Rawe et al., 2022). The elevated firing rate continues for as long as cells are illuminated (up to 1000
486 ms).
487
488 In sum, the difference between past low fractions of activated cells with ChrimsonR and the large
489 fractions of excitable cells we show here may be in part due to a difference in stimulation duration and
490 thus total current injected. Longer pulses should be expected to produce more spikes on average and
491 improve the chances of detecting photoactivation, enabling flexible targeting of behaviorally-relevant
492 neurons. One final consideration is that the calcium indicator we used may improve detection. By
493 pairing ChrimsonR with jGCaMP8s, an indicator optimized for the detection of single spikes (with the
494 tradeoff of becoming nonlinear with fewer spikes than jGCaMP8m or 8f; see Supp Fig. 6 from Zhang et
495 al., 2021), we improve our ability to detect photoactivation, while prior reports have paired ChrimsonR
496 with the less-sensitive GCaMP6 or GCaMP7 variants.
497
498 Cell responses to both optogenetic stimulation and visual stimulation are robust and stable over time
499 Many or all neurons transfected with our virus show GCaMP fluorescence throughout the cell instead of
500 being excluded from the nucleus. In single-promoter GCaMP expression, cell-filling with GCaMP is
501 often associated with a pathological state which develops over time and leads to bright fluorescence no
502 longer modulated by activity (Tian et al., 2009; Chen et al., 2013; Packer et al. 2015). In this study,
503 however, most or all neurons are filled, but the filled neurons are neither extremely bright, nor static. In
504 fact, our two-photon stimulation results (Fig. 4) and our visual stimulation results (Fig. 2) suggest that
505 the filled neurons are responding to input and reflecting these changes in GCaMP fluorescence, and
506 that our ability to measure responses is maintained over weeks (Fig. 3). Our observations of cell
507 responses to visual stimulation (Fig. 2E) and photostimulation (Extended Fig. 4-1) also match well with
508 responses measured in animals expressing jGCaMP8s and stChrimsonR via separate viruses. Visual
509 responses across the population from the different preparations are highly similar (Fig. 2E), indicating
510 many of the P2A-expressing cells respond as expected despite apparent cell-filling. Further, Tian et al.

(2009) reports that unhealthy nuclear-filling leads to an increased signal decay time, which we do not observe between the P2A and dual-virus preparations, suggesting cells expressing our P2A virus remain healthy. In fact, we find that cells expressing our P2A virus show slightly shorter decay times as compared to cells in the dual-virus preparation, and also exhibit less variance in decay times (Extended Fig. 4-1), perhaps due to less variation in relative levels of expression between opsin and indicator. The filling might arise because P2A cleavage can result in several added amino acids from the P2A linker that may yield a GCaMP protein trafficked slightly differently than the GCaMP constructs expressed with a single promoter (Kim et al., 2011). However, the neural responses we measured with this virus were robust, stable, and were similar to sensory-evoked responses previously measured in other work (Fig. 2). Therefore, if there is some difference in protein trafficking, it seems to leave the GCaMP responses to neural activity intact.

Future and conclusion

Currently, most studies employing all-optical methods for circuit dissection have focused on cortical areas. However, holographic stimulation can be used to investigate network function in other brain regions, as in the olfactory bulb (Gill et al., 2020) or the basolateral amygdala (Piantadosi et al., 2022). While viral tropisms may affect whether any given virus that works well in the cortex also works well in other brain regions, bicistronic delivery methods of both opsin and indicator will simplify testing of expression strategies.

In the present work, we build on previous efforts using separate viruses to express various GCaMP indicators (GCaMP6f, GCaMP6s, jGCaMP7s) with ChrimsonR (in mice, Attinger et al., 2017; Stamatakis et al., 2018; Chettih and Harvey, 2019; Gill et al., 2020; Daie et al., 2021; Akitake et al., 2022; O'Rawe et al., 2022; and in zebrafish, Förster et al., 2017) and on other efforts using linking peptides to simultaneously express both opsin and indicator (GCaMP6m-P2A-ChRmine, Marshel et al., 2019). Our bicistronic virus adds to the growing genetic toolbox enabling future experiments requiring

flexible patterned stimulation methods by offering a single viral approach to express the latest GCaMP8s indicator alongside the ChrimsonR opsin in cells of interest.

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Main Figure Legends

Figure 1: Widespread and stable co-expression of jGCaMP8s and stChrimsonR in mouse cortical neurons with a bicistronic viral vector.
A Schematic of the Cre-dependent vector. ***B*** Viral injections were made in primary visual cortex (V1) to achieve targeted expression in excitatory neurons using an *Emx1-Cre* mouse line; see also Extended Fig. 1-1. ***C*** Widefield fluorescence imaging through 3 mm optical windows in two example mice shows widespread expression over the cortical surface surrounding injection sites. ***D*** Two-photon calcium imaging (at 920 nm) in mouse V1 throughout layer 2/3 shows robust expression of the virus across many cells (FOV 414 x 414 µm); imaging produces minimal to no stimulation response, Extended Fig. 1-2. This figure: N = 3 mice. **C**: mouse 1-2, **D**: mouse 3.

Figure 2: V1 cells expressing jGCaMP8s-P2A-stChrimsonR show expected visually-evoked and spontaneous activity.
A Two-photon imaging FOV (414 x 414 µm) in layer 2/3 of mouse V1 expressing jGCaMP8s-P2A-stChrimsonR. Imaging: 30 Hz frame rate, 15 mW, 920 nm. ***B*** Example trial-average visual responses to a retinotopically-aligned 15 degree FWHM Gabor. One orientation shown. (In total, 8 orientations presented in random order, each 2 sec duration, N = 20 repetitions/orientation.) ***C*** Trial-average responses in four example cells showing direction and orientation tuning. DSI and OSI calculated using average $\Delta F/F_0$ across the 2 second visual period. Polar plots: responses normalized to the peak response across all 8 orientations for each cell. ***D*** $\Delta F/F_0$ traces in example cells (N = 20), showing stimulus-evoked responses across three consecutive trials. ***E*** Cumulative distribution functions showing distributions of tuning metrics and average responsivity (average response across 8 directions) for all cells from P2A mice (N = 931 cells across 3 mice, green lines) and dual-virus mice (N = 560 cells across 2 mice, orange lines). DSI: direction selectivity index, OSI: orientation selectivity index, gOSI: global orientation selectivity index. This figure: N = 5 mice. **A**-**D**: mouse 4, **E**: mouse 3, 4, 5, and two dual-virus mice.

Figure 3: V1 cells expressing jGCaMP8s-P2A-stChrimsonR maintain visual responses for weeks following injection.
A Two-photon imaging FOV (414 x 414 µm) in layer 2/3 of mouse V1. Imaging: 30 Hz frame rate, 15 mW power, 920 nm. ***B*** Example trial-average visual responses to a retinotopically-aligned Gabor (15 deg. FWHM) showing evoked activity ($\Delta F/F_0$) for a single orientation. ***C*** Cumulative distribution functions showing distributions of tuning metrics and average responsivity (average response of all 8 directions) across all cells from the mouse in **A** at two time points a month apart in the same FOV (N = 192 cells at 3 months post-injection, gray line, N = 167 cells at 4 months, black line). ***D*** Same as in **C**, but for a second mouse (N = 149 cells at 4 months post-injection, gray line, N = 175 cells at 5 months, black line). This figure: N = 2 mice. **A**-**C**: mouse 3, **D**: mouse 6.

Figure 4: Simultaneous holographic stimulation and calcium imaging in cells expressing jGCaMP8s-P2A-stChrimsonR.
A Two-photon imaging FOV (340 x 210 µm) in mouse V1, depth 122 µm from the pia. Imaging: 30 Hz frame rate, 10 mW power, 920 nm. White rings: holographic stimulation locations (N = 20). Red, gray arrows: examples of stimulated and nearby unstimulated cells, shown in panel **D**. ***B*** Responses to stimulation ($\Delta F/F_0$) in same FOV as **A** (N = 50 stim repetitions, 2.0 mW/target, 300 ms stim duration, 10 µm diameter holographic disk pattern, stim. delivered during imaging acquisition and gated off at edges of scan field, reported powers are adjusted for fast-gated duty cycle, Methods). ***C*** Trial-average activity traces for all cells (N = 89 cells). Black horizontal bar separates stimulation targets (above), other cells in the FOV (below). ***D*** Trial-average cell traces (mean ± SEM, N = 50 repetitions) for 9 example cells (N = 6 stimulated, red, and N = 3 unstimulated, gray). Errorbars (SEM) are small enough to be visible only as slightly thickened lines. Decay comparison to dual-virus expression, Ext. Fig. 4-1. ***E*** Average response map centered on targeted cells from **A**-**B**. ***F*** Average neuropil responses centered on 50 randomly selected coordinates. ***G*** Difference of maps from **E** and **F**. Horizontal black bar: cross-section in **H**. Dashed circle: 10 µm diameter holographic disk pattern. ***H*** Horizontal cross-section of response map in **G**. FWHM of response = 13.5 µm ***I***-***K*** Same as **A**-**C**, in a different FOV in the same mouse 1 month later (414 x 414 µm, depth = 130 µm, N = 50 targets, N = 348 total cells, N = 100 stim reps, 16 mW imaging power, stimulation power 2.5 mW/target, 150 ms stim duration, 10 µm diameter disk pattern). ***L*** Trial-average cell traces (N = 100 stim repetitions). Gray lines: 50 targeted cells in **I**, red line: mean ± SEM cell trace. In N = 3 animals with 10-12 stimulated target cells: 30/32 targeted cells responsive, 35/1037 unstimulated cells responsive (see Extended Figure 4-2, 4-3, 4-4). N = 4 photostimulation animals, N = 1 this figure, mouse 1. Extended Figure 4-3: N = 3 mice, mice 3, 4, 6.

Extended Figure Legends

Extended Figure 1-1: Widefield fluorescence imaging of jGCaMP8s-P2A-stChrimsonR expression.
Widefield fluorescence imaging through 3 mm optical windows in experimental mice. White stars: injection sites for each mouse (mouse 3: N = 5 sites, mouse 4: N = 10 sites, mouse 5: N = 10 sites, mouse 6: N = 6 sites). Images were acquired 22-53 days after injection.

Extended Figure 1-2: Minimal signs of crosstalk activation from resonant-galvo scanning at imaging onset.
Imaging crosstalk should lead to elevated fluorescence at onset of imaging. Cell-averaged fluorescent traces in imaging FOVs (N = 12 FOVs from N = 5 mice) across first 2 seconds of imaging do not show signs of widespread crosstalk in activation from the imaging laser (920 nm). Only a few sessions (2/12) show small increases in fluorescence over the first few seconds after imaging onset, as would be expected if imaging crosstalk were occurring (significantly higher levels of fluorescence in the 2nd second of imaging versus the 1st second $p<0.05$, Mann-Whitney U test with Bonferonni correction for multiple comparisons). On the other hand, however, four of twelve imaging sessions show significantly lower levels of fluorescence, arguing against crosstalk activation ($p<0.05$, Mann-Whitney U test with Bonferroni correction for multiple comparisons). Together, this suggests that elevated fluorescence at the start of imaging, due to crosstalk, is not a concern with this preparation.

Extended Figure 4-1: GCaMP decay times to photostimulation responses are similar between P2A and dual-virus preparations.
A Two-photon imaging FOV (414 x 414 µm) in layer 2/3 of mouse V1 for both a P2A mouse (top) and a dual-virus mouse (bottom). Imaging collected at 30 Hz frame rate, 10 mW power, 920 nm. ***B*** Trial-average photostimulation responses in 5 example cells from a P2A (left, green lines) or dual-virus (right, orange lines) mouse. Black lines: exponential decay functions fit to the 600 ms period following stimulation offset for all photostimulated cells to estimate decay times (Methods). Stimulation power was 2.0 mW/target (P2A) or 2.5 mW/target (dual-virus) for 300 ms using 10 µm-diameter disk patterns. ***C*** Half-decay times for photostimulated cells from both mice. Horizontal black lines: mean half-decay time amongst cells, vertical black lines: standard error of the mean. Half-decay times are not significantly different between P2A-expressing and dual-virus-expressing cells ($p=0.37$, 2-sample t-test). This figure: N = 2 mice. **A-C:** mouse 1 and a dual-virus mouse.

Extended Figure 4-2: Distribution of responses to photostimulation in stimulated and unstimulated populations.
A Cumulative distribution functions of photostimulation responses (from data in Fig. 4A-D) averaged over a 1 second period following stimulus onset in both stimulated cells (red) and unstimulated cells in the FOV (black). Using a 7.5% $\Delta F/F_0$ threshold, we found 19/20 stimulated cells and 5/69 unstimulated cells show reliable activation. ***B*** Same as in **A**, but for data from Fig. 4I-L. Using a 7.5% $\Delta F/F_0$ threshold, we found 33/50 stimulated cells and 45/298 unstimulated cells show reliable activation. To account for concerns of stronger neuropil contamination in response calculations when stimulating larger groups of cells, we also used a ring-based neuropil correction via Suite2p and found minimal differences in responses (35/50 stimulated cells and 41/298 unstimulated cells above a 7.5% $\Delta F/F_0$ threshold), implying neuropil signal was not contaminating cell responses. This figure: N = 1 mouse. **A-B:** mouse 1.

Extended Figure 4-3: Holographic stimulation of cells expressing jGCaMP8s-P2A-stChrimsonR.
A Two-photon imaging FOV (414 x 414 µm) in mouse V1 in 3 example mice. (Depths: 130 µm (top), 140 µm (middle), and 110 µm (bottom); each in L2/3, depth differences do not produce systematically different responses.) Imaging at 30 Hz frame rate, 15 mW imaging power, 920 nm. White dashed rings: holographic stimulation pattern (top: N = 10 targets, middle: N = 12 targets, bottom: N = 10 targets). ***B*** Corresponding stimulation response maps showing mean optogenetically-evoked activity ($\Delta F/F_0$) (N = 50 stim reps). Stimulation power 2.5 mW/target, stim duration 300 ms, 10 µm-diameter disk patterns. ***C*** Trial-average activity of all stimulated cells (light gray lines) plotted with mean ± SEM activity across all stimulated cells (red lines) and all unstimulated cells (black lines). SEM is present for unstimulated cells, but small relative to the size of the plotted line. Fraction of responsive stimulated cells: (top) 9/10, (middle) 11/12, (bottom) 10/10 (using 7.5% $\Delta F/F_0$ threshold, see Methods for details). Fraction of responsive unstimulated cells: (top) 11/436, (middle) 19/430, (bottom) 5/171.

Extended Figure 4-4: Spatial extent of photostimulation effects in unstimulated cells.
Trial-average responses to photostimulation. Red points: stimulated cells, gray points: unstimulated cells. Unstimulated cells are plotted as a function of distance to the nearest stimulated cell in the FOV. Stimulation power, duration, and the mean pairwise distance between stimulated targets is shown for all photostimulation experiments in P2A animals. This figure: N = 4 mice.