Supplementary Figure 1

Spectral properties of molecules, laser sources, and optics used in this approach.

a. Visible-wavelength regime. Shown are: fluorescence emission spectra for EGFP and EYFP (obtained from the Tsien Lab website, University of California, San Diego); the single-photon excitation spectrum for C1V1(E122T/E162T) (adapted from Yizhar et al., Nature 477, 171-178 [2011]); transmission curves for the dichroic (dark line) and emission filters (shaded areas) used in two-channel fluorescence detection (filter part numbers indicated); and the transmission curve for the long-pass laser-blocking filter (blue line; curves from Semrock). The 473 nm laser line used in single-photon excitation experiments is also indicated (dashed blue line). Each curve is normalized to its own peak value.

b. Infrared-wavelength regime. Two-photon action cross section for GCaMP3 (green) and relative C1V1 photocurrent response amplitudes (see inset) sampled at two infrared TPE center wavelengths (λ=900 nm and λ=1050 nm). Inset: sample intracellular photocurrents from illuminated HEK293T cells expressing C1V1; peak squared-intensity values were similar (2.76x10^54 J/cm^2-s^2 and 1.68x10^54 J/cm^2-s^2 at 900 nm and 1050 nm; assuming a fixed output temporal pulse-width). The GCaMP3 action cross-section was measured using fluorescence excited by focused low-power illumination (regime of quadratic power dependence) of a purified GCaMP3.3 sample (37 μM concentration in 20 mM MOPS, 100 mM KCl, 2.7 mM K2CaEGTA, at pH 7.4; R. Sun and S. S.-H. Wang, Princeton), normalized at each wavelength using side-by-side measurements of a reference fluorophore (20 µM fluorescein in water, pH 11; see Albota, M. A., Xu, C. & Webb, W., Appl. Opt. 37, 7352–7356 [1998]). C1V1 wavelength-sensitivity was evaluated at two spectral bands (λ=900 nm and 1050 nm), using whole-cell electrode recordings at constant voltage (-50 mV) in HEK293T cells transiently expressing the pLenti-CaMKIIa-C1V1(E162T)-TS-EYFP construct with focused scanning methods and an apparatus described previously (Rickgauer and Tank, PNAS 106, 15025-15030 [2009]).
Supplementary Figure 2

Schematic for either single-photon or two-photon excitation (TPE) photostimulation and TPE imaging.

a. Position of optics used to introduce the SPE source into the TPE microscope head. Abbreviations: AM, alignment mirror; FT, focusing telescope; SP, short-pass filter; DC, dichroic filter; LP, long-pass filter; PMTs, photomultiplier tubes.

b. TPE images (acquired at 920 nm) of a volume in a fluorescent plastic slide after bleaching neighboring areas using SPE (473 nm) and TPE (1064 nm, spatial focusing path; SF in Fig. 1, main text). Image intensity is inverted. Images are shown at the 1064 nm focal plane (upper) and as an xz projection of a through-focus series (lower).
Supplementary Figure 3

TPE stimulation evokes GCaMP3 transients consistent with action potentials (APs) and opsin-mediated depolarization in awake mice.

a. GCaMP3 $\Delta F/F$ values vs. stimulation pulse number. Somatic $\Delta F/F$ values for 6 neurons stimulated at 5, 10, and 20 Hz (16 ms per pulse), measured 500 ms after pulse-train onset (values for each cell are normalized to the peak response; average of 3-7 trials per data point). The monotonic relationship between $\Delta F/F$ and stimulation pulse number is consistent with a regime in which $\Delta F/F$ values also scale approximately linearly with AP number (assuming 1 AP per pulse; Tian et al., Nat. Methods 6, 875-881 [2009]). Inset: sample traces from one neuron stimulated with 10 pulses at 5, 10, and 20 Hz (each trace is a 5-trial average). Colored underlines indicate the corresponding stim. train period. Dashed line indicates the time at which values $\Delta F/F$ values were measured (500 ms after stim. onset).

b. Histogram of measured GCaMP3 fluorescence transient half-decay times following offset of a photostimulation epoch ($\tau_{1/2}$; calculated from single-exponential decay fits). Following stim. offset, transients evoked in cells returned to resting levels with off-kinetics ($\tau_{1/2} = 375\pm/196$ ms; mean +/- s.d.) in the range observed in vivo during trains of electrically stimulated APs ($\tau_{1/2} = 384\pm/76$ ms for 10 APs; Tian et al., Nat. Methods 6, 875-881 [2009]).

c. Peak GCaMP3 transient amplitude during raster-scanning photostimulation of a cell using the spatial focusing path (SF in Fig. 1, main text) shown for different TPE raster-scan periods, which varied by changing the number of lines in a raster-scan, and which were repeated over an interval of 512 ms. The dashed line indicates the approximate C1V1(t/t) inactivation time-constant ($\tau_{off} = 40-50$ ms; Mattis et al., Nat. Methods 9, 159-172 [2012]; Prakash et al., Nat. Methods 9, 1171-1179 [2012]). Faster-scanning photostimulation trials ($t_{scan} < t_{off}$) produced larger-amplitude responses than slower-scanning trials ($t_{scan} > t_{off}$; n=31 target cells; values for each cell normalized by maximum amplitude in that cell). This relationship is a signature of membrane depolarization mediated by scanning recruitment of opsin probes (Rickgauer and Tank, PNAS 106, 15025-15030 [2009]; Prakash et al., Nat. Methods 9, 1171-1179 [2012]; Packer et al., Nat. Methods 9, 1202-1205 [2012]). Inset: Exemplary $\Delta F/F$ traces from one cell illustrating this relationship (colors indicate scan periods of same-color dots in panel; bars indicate s.d.).