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**All-optical neuronal circuit interrogation
with reduced crosstalk between imaging
and photostimulation**

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Abstract

All-optical two-photon (2P) methods are considered of great importance to causally investigate neuronal circuits underlying mammalian behavior with near cellular resolution. These approaches typically involve a combination of green indicators (e.g., GCaMPs) and red-light-sensitive opsins (e.g., C1V1, ChRmine). However, red-shifted opsins typically display a blue-shifted tail in their absorption spectra, partially overlapping with the absorption spectra of green indicators. This overlap may lead to unwanted neuronal perturbation during imaging periods (Packer et al., 2015; Rickgauer & Tank, 2009), resulting in biased results. Here, we developed an all-optical 2P preparation combining high-efficiency two-photon manipulation using the large-conductance, blue-light-sensitive opsin stCoChR (Forli et al., 2021) and two-photon imaging using the high-dynamic range red-shifted indicator RCaMP3 (Yokoyama et al., 2024) in layer 2/3 pyramidal neurons of primary somatosensory cortex (S1) of awake head-fixed mice. With this experimental strategy, we achieved: i) recording of network activity with a larger dynamic range and sensitivity compared to previously used red-shifted indicators; ii) effective photo-activation of neurons with low average power *per* cell; iii) reduced crosstalk between imaging and photostimulation; iv) functional co-expression of the opsin and indicator with a bicistronic construct developed in collaboration with Prof. Sakamoto, Kyoto University; v) investigation of the influence of local network dynamics and animal arousal level on the two-photon photostimulation neuronal responses. This novel experimental approach will enable high-efficiency recording and manipulation of neuronal networks *in vivo*, while granting effective spectral separation between the excitation of the opsin and of the calcium indicator.

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1. Introduction

1.1 Linking neuronal activity to behavior

Neuroscience has long revolved around the study of individual neurons, posited to be the structural and functional unit of the nervous system by Cajal and Sherrington in the late XIX and early XX centuries (Ramón y Cajal, 1888; Shepherd, 2016; Sherrington, 1906). Initially confined to anatomical descriptions and Sherrington's works on arc reflexes, this "neuron doctrine" was consolidated in neuroscience with the introduction of techniques allowing functional studies of single neurons, which revealed their biophysical properties and characterized their responses to sensory stimuli (Adrian, 1928, 1947; Barlow, 1953; Hubel, 1957; Hubel & Wiesel, 1962). Accumulated evidence indicates a close relationship between the electrical activity of single cells and various behavioral parameters across areas and species, particularly with behavioral features related to sensory perception (Barlow, 1972; Brecht et al., 2004; Desimone et al., 1984; Houweling & Brecht, 2008; Kreiman et al., 2000; Salzman & Newsome, 1994; Tanaka, 1996). However, neurons can have different anatomical, transcriptional, electrical, and chemical profiles (Gouwens et al., 2019; Liu et al., 2024; Yao et al., 2023); they are organized in the three spatial dimensions, connected over micro-, meso-, and macro-scales (Kandel et al., 2013); they code information into spike rate and spike timing (Bialek et al., 1991; London et al., 2010; Rieke, 1999). All these different features converge to form architecturally complex neuronal circuits, whose dynamics generate sub-networks that display specific spatio-temporal patterns of neural activity. With the advent of optical imaging methods and multielectrode recordings, spatio-temporal activity patterns in populations of neurons, rather than single cells' activity in isolation, are increasingly recognized as the key physiological phenomenon in neuronal processing underlying many brain different processes

and states (Buonomano & Maass, 2009; Buzsáki, 2010; Eichenbaum, 2018; Saxena & Cunningham, 2019). Within this context, a population of neurons displaying “recurring patterns of endogenously coordinated activity” (Yuste et al., 2024), whether this is linked to an external stimulus or to an internal state, is commonly termed “ensemble”. Ensemble activity has been associated with different behavioral and cognitive domains, such as sensory perception (Carrillo-Reid et al., 2019; Dal Maschio et al., 2017), motor actions (Peters et al., 2014), learning and memory (Tanaka et al., 2018), feeding and social behaviors (de Araujo et al., 2006; Jennings et al., 2019), as well as internal states (Gründemann et al., 2019). To properly address the function of neuronal ensembles and uncover how the spatial and temporal structure of network activation is linked to specific behaviors, cognitive functions, or pathologies, neuroscientists require tools capable of simultaneously recording and manipulating the activity of multiple neurons with high spatiotemporal resolution *in vivo*. The ability to identify activity patterns correlated with sensory stimuli or behavioral outputs, and to subsequently bias these patterns, would enable rigorous causal probing of the functional significance of neuronal ensembles. In the context of sensory processing, this could generate important insights into how information contributes to the encoding of a stimulus (i.e., how a given stimulus is represented in a specific area or circuit in the brain in the form of spatiotemporal patterns of activity) or how information contributes to the decoding of a choice or an action (i.e., how encoded information is used to instruct an appropriate behavioral response, (Panzeri et al., 2017).

1.2 Recording neuronal activity

Monitoring the activity of specific groups of neurons related to complex behaviors such as sensory perception or motor action *in vivo* is an essential yet challenging task in neuroscience. In fact, neuronal activity has temporal dynamics that span several orders of magnitude, from milliseconds to days. The spatial scales over which neuronal activity is organized similarly span several orders of magnitude, from micrometers to meters. Virtually any brain region in the mammalian brain displays this level of complexity at both the anatomical and functional level (Harris et al., 2016). For example, one common model system in neuroscience is the primary somatosensory cortex of rodents, particularly the somatotopically-organized barrel field, implicated in the processing of somatosensory stimuli collected by the whiskers (Brecht, 2007; Petersen, 2007). This area is a prototypical experimental system for investigating interactions among neurons within a sensory cortical area and its communication with other cortical and subcortical regions. Despite its seemingly simple organization, the barrel field displays 33 cylindrical modules, each comprising ~10,000 neurons in a 300 x 300 x 900 μm volume (Feldmeyer et al., 2013). Every barrel can be segregated into distinct functional or anatomical subgroups, each with its unique electrophysiological and connectional profiles (Staiger & Petersen, 2021). These non-homogeneously distributed excitatory and inhibitory neurons are connected to tens or even hundreds of other neurons via micrometer-scale processes and communicate through brief electrical and chemical signals lasting a few milliseconds (Helmstaedter et al., 2007). Considering the whole cortex of the adult mouse, namely a 112 mm^3 volume composed of ~14 million neurons, it is clear that to successfully record neuronal activity, a non-invasive, spatiotemporally precise method is essential (Carrillo-Reid & Yuste, 2020; Packer et al., 2013; Schüz et al., 2006; Schüz & Palm, 1989). Such a

method would provide important information about both the “where” and the “when” signals correlated with behavioral measures are generated.

1.2.1 Electrophysiological recordings

Electrophysiology has long been the gold standard method to record neuronal activity. The method has provided the first experimental evidence of electrical currents generation in the brain (Caton, 1875), their propagation over sensory nerve fibers (Adrian & Zotterman, 1926), as well as the first description of brain electrical rhythms (Berger, 1875). Intracellular recordings, in particular the voltage-clamp (Hodgkin & Huxley, 1939; Figure 1) and the patch-clamp techniques (Sakmann & Neher, 1984), have yielded invaluable information about the biophysical properties of neurons. These techniques enabled the study of sub- and supra-threshold electrical activity, and the flow of ionic currents through membrane channels, respectively.

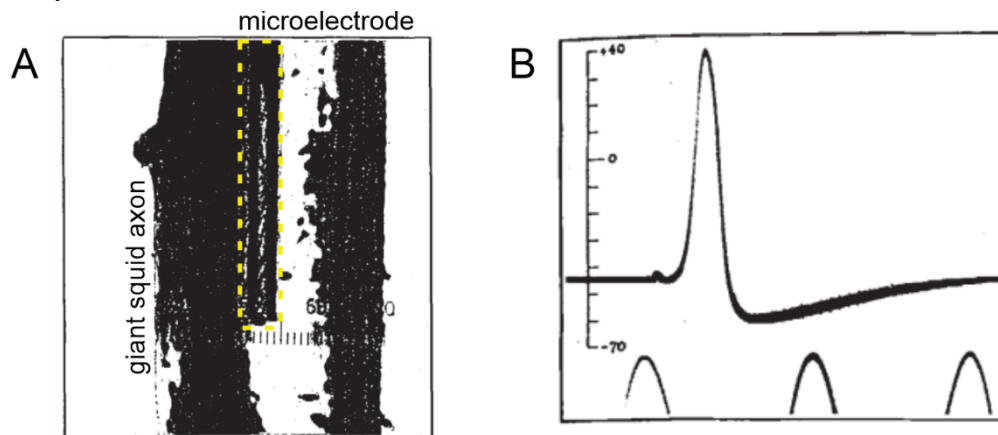


Figure 1. Electrophysiological recordings contributed to reveal neuronal biophysics

A. Microphotograph of the electrode (highlighted in yellow) inserted into the giant squid axon; **B.** The first action potential recorded intracellularly from a neuron. Adapted from (Hodgkin & Huxley, 1939).

Single-cell recordings also established the first links between patterns of electrical activity in single cells and specific external stimuli *in vivo* (e.g., Adrian & Zotterman, 1926; Hubel &

Wiesel, 1962). Different aspects of these responses have been considered to explain the encoding of sensory information, including spike rate (London et al., 2010), spike timing (Ermentrout et al., 2008; Panzeri et al., 2001; Stein et al., 2005), and spike timing variability (Arieli et al., 1996). However, the idea that the brain would encode sensory information in isolated cells, whether in a “rate code” or a “temporal code”, is challenging to accept. Both cell mortality and the need to propagate signals robust to noise, for example, argue against it (Pouget et al., 2000). An alternative hypothesis is that of “population coding”. Rooted in Hebb’s cell assembly theory (Hebb, 1949) and Hopfield network models (Hopfield, 1982; Hopfield & Tank, 1985), this theory assumes that behavioral and cognitive functions are emergent properties of the combined activity of multiple cells acting together, rather than single neurons’ activity in isolation (Averbeck et al., 2006; Pouget et al., 2000). This view does not exclude nor resolve the question of whether and how rate or temporal aspects of spike patterns are important in behaviorally relevant stimuli and actions, but rather emphasizes the importance of the combination of activity from multiple neurons and their anatomical and connectional specificities in the processing of information. As neural activity is distributed over populations of cells spread over different spatial scales, significant efforts have been made to establish extracellular measurements from populations of neurons rather than single cells in isolation. These efforts have led to the development of multi-electrode arrays (e.g., Maynard et al., 1997; Thomas et al., 1972), which nowadays allow the experimenter to simultaneously record hundreds of single units from a single shank (Angotzi et al., 2024; Berényi et al., 2014; Jun et al., 2017; Steinmetz et al., 2021), describe the interactions between the recorded neurons, and infer potential mechanistic explanations. For example, the use of these tools has shown that response variability correlates between neurons, although this variability was initially

considered noise (Cohen & Kohn, 2011; Shadlen & Newsome, 1998). As these tools allow for monitoring head-fixed as well as freely moving animals over long time windows (days to weeks), potentially from different brain areas, they are well-suited for use in studies employing behavioral tasks. Also, multiplexing the number of shanks inserted into one single brain has made it possible to study rhythmic oscillations or correlated activity between areas (e.g., Stringer et al., 2019). Electrophysiological recordings grant direct access to the electrical activity of cells with unparalleled temporal precision, but suffer from several limitations. Intracellular recordings *in vivo* damage the tissue upon insertion, have a limited yield in terms of the number of neurons recorded, and are not suited for prolonged measurements due to dilution of cytoplasmic content. One slightly less invasive technique in this context is the cell-attached (juxtosomal) extracellular recording (Kitamura et al., 2008; Margrie et al., 2002), which does not penetrate the neuronal membrane, allowing the measurement of suprathreshold events (i.e., action potentials) only. Other extracellular recording configurations, such as local field potential and multi-unit activity, address two of the limitations of the aforementioned methods, allowing prolonged, repeated measurements from large populations of neurons, though they remain invasive recording techniques. Technological advancements in electrode miniaturization procedures (Keren & Hanein, 2013; Spira & Hai, 2013) have provided higher sampling density and biocompatibility, while new analytical methods optimize data extraction and analysis (e.g., Pachitariu et al., 2024; Stringer et al., 2025). However, these methods are inherently affected by low spatial resolution - typically, increasing spatial resolution in extracellular recordings often comes at the expense of the area of signal integration - and maintaining viable recordings from the same cells remains a challenging quest. Also, electrophysiological population measurements do not target specific neuronal populations,

although optogenetic tagging has been combined with electrophysiology (Cardin et al., 2010; J. Y. Cohen et al., 2012; Lima & Miesenböck, 2005). Other extracellular recording methods include the electrocorticogram (ECoG) and the electroencephalogram (EEG). While EEG is the only truly non-invasive technology listed here, neither of these tools requires parenchymal penetration. However, the spatial resolution of EEG and ECoG is lower than that of other extracellular recording methods, as they typically collect signals integrated over millimeters of brain tissue and that primarily originate in the cortex (Buzsáki et al., 2012; Nunez & Srinivasan, 2006).

1.2.2 Optical recordings

Recordings of neuronal population activity patterns should ideally achieve high spatial and temporal resolution, non-invasively and in vivo, allowing the observation of ongoing dynamics in preserved neural circuits and correlating their functional signatures with their anatomical location and behavioral output. Two technical advances have been crucial to achieve this goal, providing limited invasiveness and high spatial resolution: scanning fluorescence microscopy (particularly non-linear microscopy) and the development of fluorescent activity reporters. In the last three decades, the combination of these two technologies paved the way for optical imaging methods to become a preferred tool to record the activity of neuronal populations (from tens to tens of thousands of cells) in the intact brain with cellular resolution (Bovetti et al., 2014; Grienberger & Konnerth, 2012; Svoboda & Yasuda, 2006; Yang & Yuste, 2017; Yuste & Denk, 1995; Yuste & Katz, 1991). Optical recordings are generally minimally invasive, only requiring the substitution of a cranial bone flap with an optical window to provide light access to the underlying tissue (Holtmaat et al., 2009). Light does not destroy or damage it, provided

that the average light power is below the photodamage threshold (Podgorski et al., 2016). However, the brain parenchyma is a highly scattering medium, ultimately limiting light penetration to a maximum of ~800 microns with 2P microscopy (Helmchen & Denk, 2005). To access deeper anatomical structures with 2P microscopy, light must be relayed farther into the encephalon with invasive endoscopic probes (e.g., gradient refractive index (GRIN) lenses; (Barretto et al., 2009) that allow the focal plane of the microscope objective to be relayed down to the deeper brain region of interest. Such devices must be inserted into the tissue, limiting the field of view dimension and potentially triggering inflammation in the surrounding area.

Fluorescence is the spontaneous emission of one photon by a molecule (called a fluorophore), which has been previously excited by illumination with light of the appropriate wavelength and intensity. When a fluorophore molecule absorbs one or more photons, its electrons transition from the ground energy state to a higher energy level, the excited state. Electrons subsequently relax back to the ground state and release energy in the form of emitted fluorescence photons. These emitted photons have lower energy and longer wavelengths than the absorbed ones, due to energy loss during the relaxation process (Pawley, 2006). In standard one-photon (1P) wide-field fluorescence microscopy, the fluorescence signal linearly depends on the intensity of the incident light. However, when employed to illuminate highly scattering media such as the brain, the image quality rapidly decreases because of the high sensitivity to scattering of the visible light wavelength used in 1P fluorescence microscopy and the out-of-focus excitation that is intrinsic to 1P fluorescence microscopy. Non-linear two-photon (2P) fluorescence microscopy has partially addressed both these issues. In the two-photon excitation process, first hypothesized by Marie Goeppert-Mayer in 1931 (Göppert-Mayer, 1931), the

transition of one electron from the ground state to the excited state is mediated by two excitation photons absorbed “quasi-simultaneously” (within ~ 0.5 femtoseconds) by the molecule. In the degenerate multiphoton absorption process (He, 2022), each photon carries half the energy and has, therefore, twice the wavelength of photons needed in 1P fluorescence excitation (Svoboda & Yasuda, 2006; Figure 2A). Because two-photon excitation employs longer wavelengths and the brain scattering coefficient decreases with wavelength, sensitivity to scattering is reduced, and deeper structures (up to $\sim 800 \mu\text{m}$) can be reached (Helmchen & Denk, 2005). In fact, most mammalian tissues exhibit maximal transparency to light within the wavelength range of ~ 600 to ~ 1300 nanometers -i.e., the range between the absorption spectra of hemoglobin and water, respectively (Lyons et al., 2013; Tromberg et al., 2000). Using near-infrared (IR) wavelengths within this “optical window” (600-1300 nm) ensures that excitation light penetrates deeper within the tissue. Moreover, the 2P excitation process depends on the second power of the light intensity. This, in combination with high numerical aperture (NA) objectives, grants localized excitation of fluorophores only in the diffraction-limited volume of focused light where photon density is higher, leading to a much-improved excitation resolution compared to the standard 1P excitation process (Svoboda et al., 1997). Since 2P excitation has inherently low probability, it requires high temporal confinement of excitation photons, typically provided by ultrafast pulsed lasers (Denk et al., 1990; Figure 2C). High photon densities in space are achieved by focusing coherent light through high-NA objectives. Under diffraction-limited conditions, the focal spot size (w_0) at the focal plane scales as $w_0 \approx \lambda/(2\text{NA})$, indicating that higher NA values grant tighter, more confined spatial focusing of photons at the sample.

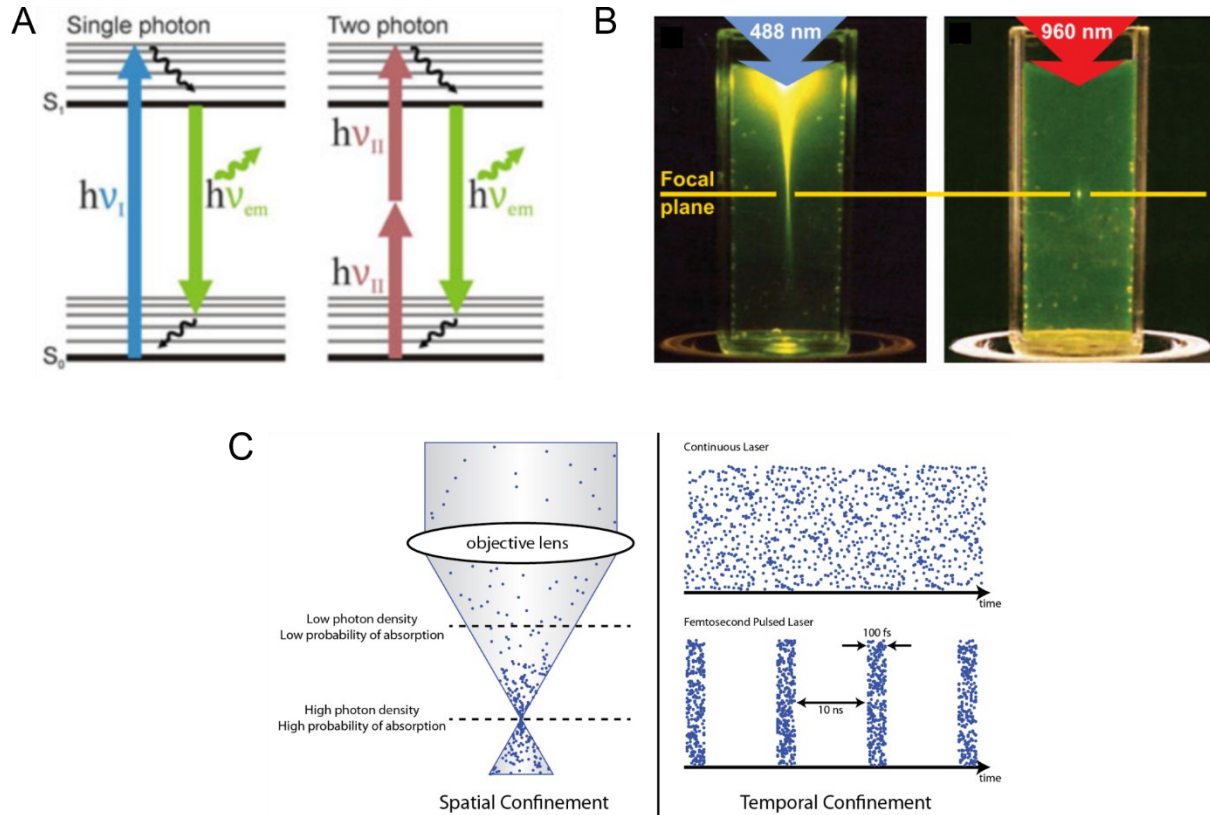


Figure 2. 1P fluorescence excitation vs 2P fluorescence excitation.

A. Jablonski diagram depicting the transitions of an electron from the ground energy state S_0 to the excited energy state S_1 following the absorption of one photon (energy $h\nu$, left) or two quasi-simultaneous photons of half the energy ($h\nu'$, red arrow, right). The blue arrow represents the transition after one-photon absorption, the two red arrows represent the two-photon excitation process. In both cases, one photon is emitted during the relaxation phase toward the ground state (green arrows). **B.** Excitation volume for 1P (left) and 2P (right) excitation in a cuvette filled with fluorescein using the same objective lens. **C.** Spatial and temporal confinement of photons (dots) by means of an appropriate objective lens and a femtosecond pulse laser. Efficient 2P excitation is generated only at the focal plane with minimal out-of-focus multiphoton absorption. Adapted from (A, Dal Maschio et al., 2012; B, Zipfel et al., 2003; C, <http://sites.middlebury.edu/durst/research/>).

The most prevalent reporters of neuronal activity to be coupled with functional fluorescence microscopy are calcium sensors. Action potentials in neurons are associated with the opening of voltage-gated calcium channels, which lead to an inflow of calcium ions along the electrochemical gradient inside the cell, raising its cytosolic calcium concentration (Augustine et al., 1987; Jaffe et al., 1992). Fluorescent calcium sensors are fluorescent proteins that

undergo conformational changes upon calcium binding, resulting in a change in their fluorescence emission (Miyawaki et al., 1997a; Wemmer et al., 1985; Yamada et al., 1995). By monitoring the changes over time of the emitted fluorescence of fluorescent calcium sensors in the cell bodies it is therefore possible to indirectly measure the suprathreshold activity (APs) of neuronal cells (Denk et al., 1996; Yasuda et al., 2004). As fluorescent calcium indicators report changes in calcium concentration rather than direct electrical activity, the resulting fluorescence signals intrinsically cover slower temporal scales (in the order of hundreds of milliseconds in most cases) compared to the actual electrophysiological activity of neurons (Harris et al., 2016; Nakai et al., 2001). Moreover, calcium sensors kinetics and subcellular localization further slow down the temporal response of the fluorescence signal, although the newer generation of calcium indicators displays faster kinetics (Chen et al., 2013; Dana et al., 2019; Y. Zhang et al., 2023). For this reason, while most neuronal cell types can be monitored with the newest generations of calcium indicators, their use with fast-spiking cells remains challenging (Dana et al., 2019; Zhang et al., 2023).

Originally, calcium sensors were developed as synthetic dyes (e.g., Grynkiewicz et al., 1985; Tsien, 1980) and saw widespread adoption. Using synthetic dyes, two-photon calcium imaging of neurons was achieved in brain slices (Göbel et al., 2007; Yuste & Denk, 1995; Yuste & Katz, 1991) as well as *in vivo*, opening a new era of neuroscientific investigation (Ohki et al., 2005; Stosiek et al., 2003; Svoboda et al., 1997; Figure 3).

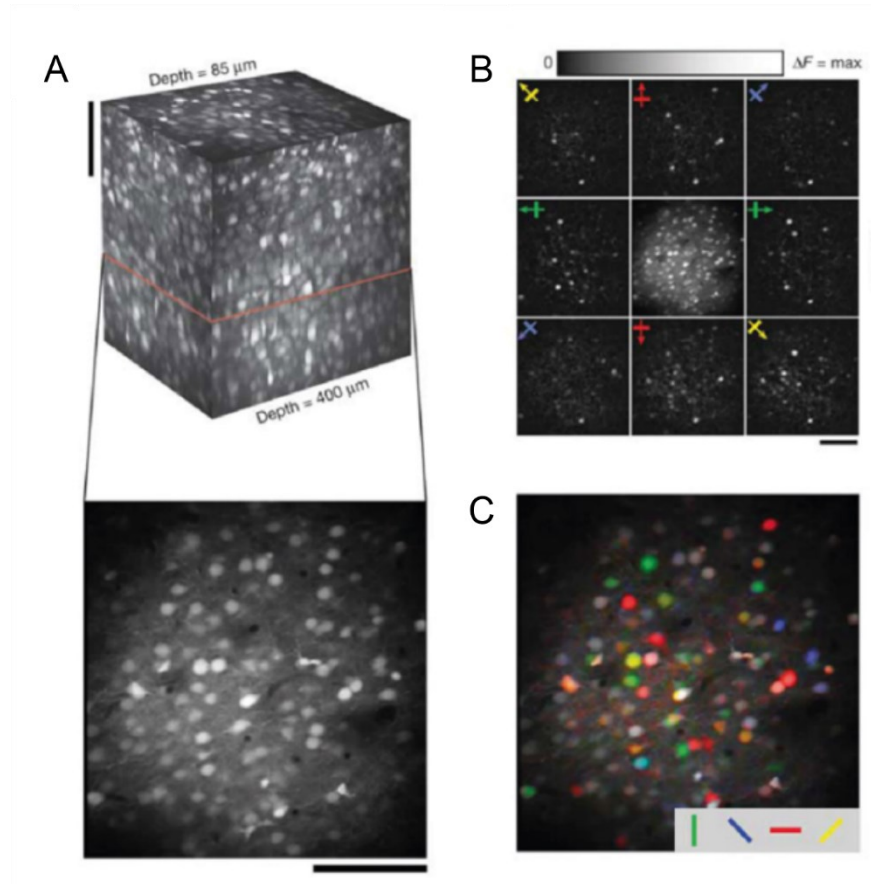


Figure 3. *In vivo* 2P calcium imaging of rat visual cortex reveals single-cell resolution maps of orientation-selective neurons.

A. *In vivo* images of cortical cells stained with the synthetic calcium indicator OGB-1 AM. Top: volume of stained cells (85–400 μm depth) reconstructed from images spaced 1 μm; Bottom: anatomical image at 290 μm below the pia, averaged over all frames during the visual stimuli presentation protocol. **B.** Single-condition maps (ΔF) for eight directions of visual stimuli (colored arrows indicate stimulus direction). **C.** Pixel-based orientation map: color is determined by the response to the preferred orientation and is overlaid with the anatomical image in (A).

However, synthetic dyes presented drawbacks: they labelled neurons non-specifically, had limited lifetime once expressed, precluding chronic measures (Hendel et al., 2008; Mank et al., 2008; Stosiek et al., 2003). An important advancement that allowed to overcome these limitations was the introduction of genetically encoded calcium indicators (GECIs; Miyawaki et al., 1997). These indicators are protein produced directly by genetically targeted cells. GECIs have enabled cell-type-specific and long-term monitoring of neuronal activity.

Combined with 2P imaging, GECIs fundamentally contributed the investigation of the neural correlates of behavioral tasks (Grienberger & Konnerth, 2012; Knöpfel et al., 2006). These sensors can be expressed via different genetic strategies but typically require more time (typically >10 days) to reach the level of expression compatible with two-photon imaging compared to synthetic dyes (typically in the order of hours) and can present variability in expression levels (Akerboom et al., 2009, 2013; Chen et al., 2013; Dana et al., 2016, 2019; Inoue et al., 2015; Mohr et al., 2020; Nakai et al., 2001; Tian et al., 2009; Zhang et al., 2023). Common expression approaches are *in-utero* electroporation, intraparenchymal injection of engineered viral vectors (e.g., adeno-associated viruses, AAV), noninvasive AAV-PhP intravenous injections (Challis et al., 2019; Chan et al., 2017), or transgenic animal lines constitutively expressing the indicator in a selected neuronal population (Madisen et al., 2010; Dal Maschio et al., 2012). In AAV injections, the coding sequence of the fluorescent indicator is inserted into plasmid backbones downstream of an appropriate cell-type-specific promoters. Once infected with the AAV, the host cell transcription and translation machinery expresses the coded protein. Infection efficiency and cell-type tropism may depend on the serotype of the virus (Aschauer et al., 2013; Lee et al., 2017; Powell et al., 2020; Zincarelli et al., 2008). Moreover, cell-type specificity can be enhanced by using cell-type-selective promoters or conditional systems. For example, Cre-lox recombination strategies restrict the expression of a floxed transgene to Cre-expressing cells: the transgene is flanked by loxP sites and remains inactive until Cre recombinase mediates sequence inversion, enabling correct transcription (Sternberg & Hamilton, 1981). This method is important because it decouples cell-type specificity from expression level. A weak but highly cell-specific promoter can be used to express the Cre, while the floxed gene is controlled by a strong but cell-non-specific promoter

(Kuhlman & Huang, 2008; Madisen et al., 2010, 2012; Sohal et al., 2009). The most widely adopted GECIs belong to the GCaMP family (Nakai et al., 2001), which incorporates the circularly permuted green fluorescent protein (GFP) to report calcium transients with high sensitivity. Iterative improvements have enhanced their dynamic range, kinetics, and signal-to-noise ratio (Akerboom et al., 2009; Chen et al., 2013; Dana et al., 2019; Tian et al., 2009; Zhang et al., 2023; Figure 4).

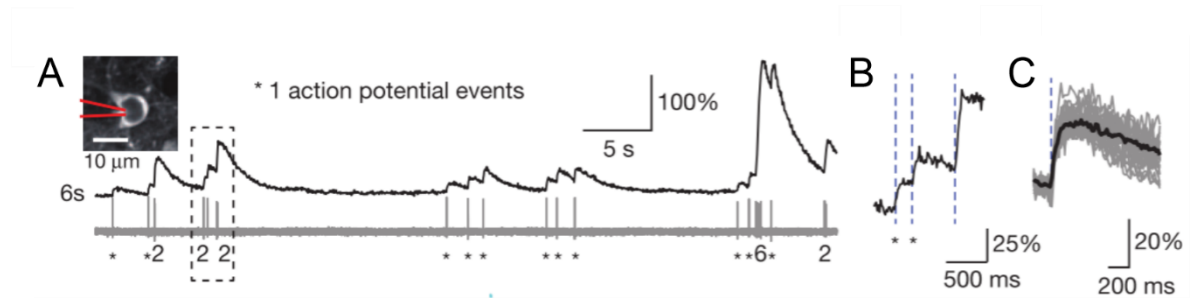


Figure 4. Spontaneous neuronal activity in layer 2/3 pyramidal cells of primary visual cortex recorded *in vivo* with calcium indicator GCaMP6s.

A. Simultaneous recordings of fluorescent dynamics (top) and spiking activity (bottom) from a GCaMP6s-expressing neuron; the exact number of action potentials fired is indicated below the electrophysiology trace. Left inset: GCaMP6s-expressing neuron recorded in the cell-attached configuration. **B.** Calcium dynamics in correspondence with a burst of action potentials ($n = 3$ APs), single recording. **C.** Averaged fluorescence response from multiple recordings to a single action potential. Adapted from (Chen et al., 2013).

Other GECIs with different spectral properties and kinetics have been developed. For example, RCaMPs, GECOs, jRCaMPs, jRGECOs, and jYCaMPs are optimized to absorb far-infrared light above 1040 nm (Akerboom et al., 2013; Dalangin et al., 2025; Dana et al., 2016; Inoue et al., 2015, 2019; Mohr et al., 2020; Yokoyama et al., 2024; Zhao et al., 2011). This variety of fluorescent reporters can serve different experimental needs: for example, expressing indicators with different spectral properties (e.g., GCaMP and RCaMP) in different cell populations or different subcellular compartments enables spectrally multiplexed investigation

of cellular interactions (e.g., Akerboom et al., 2013; Curreli et al., 2022; Meng et al., 2018; Sakamoto & Yokoyama, 2025); alternatively, the co-expression of a calcium reporter with an actuator that absorbs light at a different wavelength permits all-optical studies combining imaging and optogenetics (Bounds et al., 2023; Chettih & Harvey, 2019; Daie et al., 2021; Gill et al., 2020; Jennings et al., 2019; Mardinly et al., 2018; Marshel et al., 2019; Oldenburg et al., 2024; Packer et al., 2015; Rickgauer & Tank, 2009; Russell et al., 2024; Wilson et al., 2012; Zhang & Oertner, 2007). The GCaMP family has undergone tremendous improvements over the years, while red- or yellow-shifted indicators have remained inferior in terms of kinetics and sensitivity compared to green-emitting sensors. For example, jRGECO1a (Dana et al., 2016) has long been one of the most commonly used red GECI, but it shows three times dimmer fluorescence changes compared to GCaMP6s (Chen et al., 2013). These limitations have been recently addressed with the development of two new promising, red-shifted indicators: RCaMP3, developed from mutated forms of jRGECO1a, and PinkyCaMP, a newly engineered mScarlet-based indicator (Fink et al., 2024; Yokoyama et al., 2024). PinkyCaMP has improved brightness and photostability upon blue-light illumination compared to RCaMP3. The latter, instead, has higher $\Delta F/F_0$ and faster decay times. Both indicators serve as valuable tools for dual-color experiments that employ another green-shifted indicator to simultaneously image different biological signals (Figure 5). They are also suited to be coupled with blue-shifted opsins to enable 2P all-optical experiments: this experimental strategy allows to selectively and simultaneously photostimulate multiple genetically identified neurons with *quasi*-single cell resolution while recording their activity through calcium imaging with minimized crosstalk (Forli et al., 2018, 2021; Packer et al., 2015; Venkatachalam & Cohen, 2014).

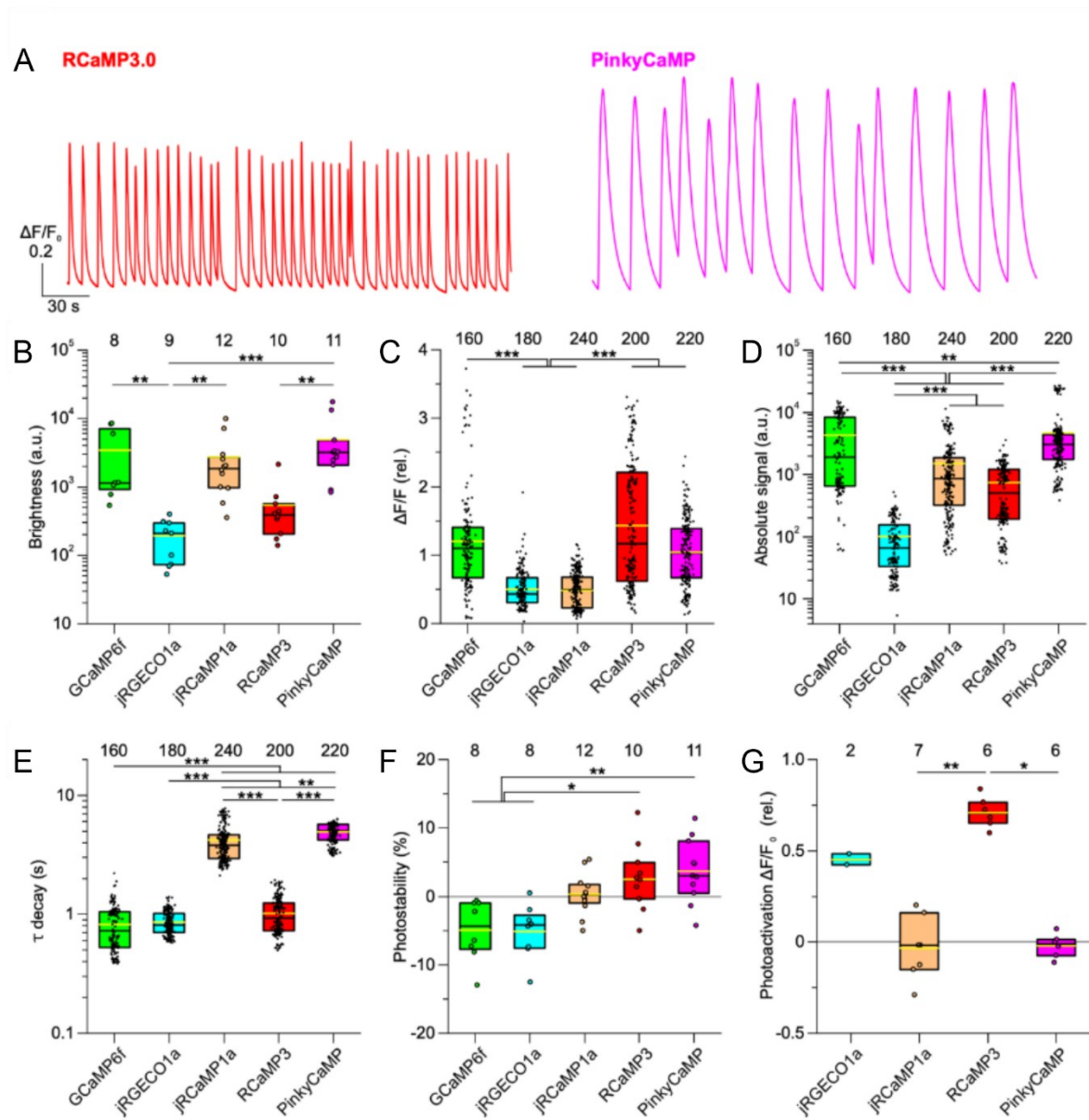


Figure 5. Comparison of RCaMP3 and PinkyCaMP and other calcium sensors.

A, B. Spontaneous, synchronous calcium transients recorded with RCaMP3 and PinkyCaMP in slice cultures from the mouse cortex. Data for GCaMP6f, jRGECO1a, jRCaMP1a, RCaMP3, and PinkyCaMP are reported: **C.** Brightness (baseline fluorescence, F_0); **D.** Relative signal change ($\Delta F/F_0$); **E.** Absolute brightness (F_0 over $\Delta F/F_0$); **F.** Decay time constants; **G.** Photobleaching; **H.** Photoactivation upon blue light illumination. Black boxes indicate 25-75% percentiles and medians, yellow lines indicate means. The number of analyzed slices (C, G, H) or events (D, E, F) is given. Statistical differences are indicated with * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$. Adapted from (Fink et al., 2024).

A promising alternative to GECIs are genetically encoded voltage indicators (GEVIs; Abdelfattah et al., 2019; Adam et al., 2019; Knöpfel & Song, 2019; Xu et al., 2017), light-emitting reporters of neuronal activity that can directly report supra- and sub-threshold membrane potential changes in neurons. GEVIs provide a 100- to 1000-fold faster temporal resolution compared to calcium sensors, but generally show dim fluorescence (Yang & St-Pierre, 2016). GEVIs require fast (kHz range) acquisition rates and generally high illumination powers -possibly leading to photobleaching. Moreover, sparse expression levels of GECI are required to avoid cross-cell fluorescence contamination (Lin & Schnitzer, 2016; Yang & St-Pierre, 2016). Due to these constraints, GEVIs are not yet the preferred tool for population-level two-photon recordings *in vivo* over long time windows (Sakamoto & Yokoyama, 2025).

1.3 Optogenetics

Performing 2P calcium imaging of neuronal activity during behavioral tasks *in vivo* provides important information about the correlation between the activity of specific neuronal subpopulations and specific parameter of the animal behavior during the task. However, understanding causal links between neuronal activity and behavior requires the ability not only to monitor neuronal activity but also to manipulate the cellular activity patterns and measure the impact of this manipulation at the network and behavioral levels (Emiliani et al., 2015; Panzeri et al., 2017; Russell et al., 2022). In the past, the manipulation of the activity of certain brain areas was provided by electrical stimulation (Bliss & Lomo, 1973; Galvani, 1786; Penfield & Boldrey, 1937) or pharmacological intervention (Carlsson & Lindqvist, 1963; Nader et al., 2002). Both approaches, though, are not suited for single-cell, circuit-level manipulations (Cohen & Newsome, 2004; Histed et al., 2009; Lee et al., 2020). When

designing a perturbational experiment, ideally, the experimenter should be able to tailor the intervention based on a multitude of parameters: the specific neuronal population under investigation, its spatial distribution, its connectivity profile, its spiking rate, the precise timing of spikes in response to stimuli, locomotor activity or internal state changes of the animal, the role specific cells play in the encoding of a stimulus or the decoding of an appropriate behavioral response, what is the role of the cell within its local network, and so on (Adesnik & Abdeladim, 2021; Russell et al., 2022). Neither electrical stimulation nor pharmacological intervention satisfy these prerequisites. For example, the first lacks cellular specificity, and the second lacks temporal precision. In 1999, Francis Crick proposed: "one of the next requirements [...] is to be able to turn the firing of one or more types of neuron on and off in the alert animal in a rapid manner. The ideal signal would be light, probably at an infrared wavelength, to allow the light to penetrate far enough. This seems rather far-fetched, but it is conceivable that molecular biologists could engineer a particular cell type to be sensitive to light in this way." (Crick, 1999) Six years later such a method was devised: optogenetics (Boyden et al., 2005; Lima & Miesenböck, 2005). Optogenetics combines genetic manipulation of cell types to make them sensitive to light with optical methods to deliver the light that will allow their stimulation or inhibition, with high temporal and, depending on the illumination method, spatial precision, in living animals (Deisseroth, 2015; Yizhar et al., 2011)

1.3.1 Optogenetics: opsins

Light-dependent processes occur in many living forms, especially algae, bacteria, and fungi (Ernst et al., 2013). These organisms use photoreceptors for capturing and storing energy, phototaxis, environmental responses, and reproduction (Brown, 2004; Foster et al., 1984;

Spudich, 2006; Zhang et al., 2011). Light-sensitive proteins involved in these processes have been studied for a long time, but only in the early 2000s have they started to be used to engineer naturally light-insensitive cells into light-sensitive ones (Boyden, 2011; Nagel et al., 2002a, 2003; Zemelman et al., 2002, 2003). These proteins (also known as opsins or actuators, in this context) typically contain a chromophore (e.g., the retinal group), which confers the ability to absorb photon of appropriate wavelength. Upon light absorption, the retinal group typically undergoes a cis-to-trans- photoisomerization, which induces a conformation change in the opsin that enables the translocation of ions across the cell membrane (if the opsin is a light-gated transporter) or the opening of a pore through which ions flow along their electrochemical gradient (if the opsin is a light-gated channel) (Ernst et al., 2013; Govorunova et al., 2017; Zhang et al., 2011). Naturally occurring opsins combined with appropriate control elements (promoters, targeting sequences) allow for cell-type-specific expression of such proteins into specific target cells in the mammalian brain (Atasoy et al., 2008; Cardin et al., 2010; Zhang & Oertner, 2007). There are multiple types of opsins (Hegemann, 2008). Microbial opsins (Figure 6), or type I opsins (Govorunova et al., 2017; Rozenberg et al., 2021), are the most extensively used in neuroscience. They are ion channels or ion transporters that covalently bind all-trans retinal and, upon illumination, allow ion flux or translocation across the plasma membrane (Deisseroth & Hegemann, 2017; Ernst et al., 2014). Microbial opsins can be further subdivided into three classes: channelrhodopsins, halorhodopsins, and bacteriorhodopsins (Grote et al., 2014; Zhang et al., 2011).

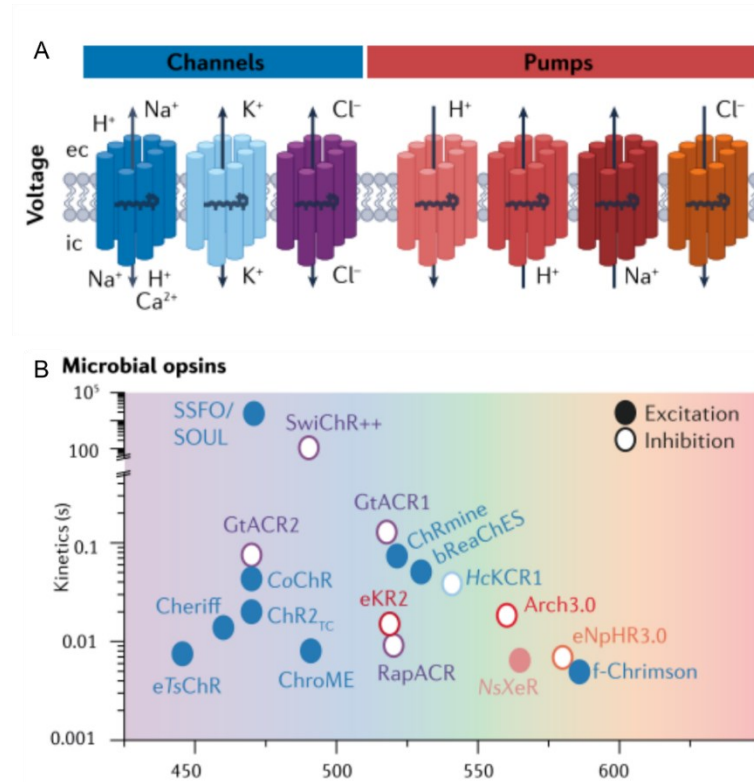


Figure 6. Light-activated ion channels and pumps used in optogenetics

A. Schematic representation of microbial rhodopsins, categorized by their function as ion channels (blue) or ion pumps (red). Channels facilitate passive ion flow along electrochemical gradients; ion pumps actively transport ions against their gradients using light energy. Channels include: H^+ channel (e.g., Channelrhodopsin or engineered variants), Na^+ channel (e.g., Na^+ -selective ChRs variants), K^+ channel (e.g., BLINK1), Cl^- channel (e.g., GtACR1/2). Pumps include: H^+ pumps (e.g., Bacteriorhodopsin and Archaeorhodopsin), Na^+ pump (e.g., KR2), Cl^- pump (e.g., Halorhodopsins). Arrows indicate the direction of ion movement from intracellular (ic) to extracellular (ec) space and viceversa depending on the electrochemical gradient of the specific ion under consideration. **B.** Commonly used excitatory and inhibitory opsins of neuronal activity, plotted according to peak excitation wavelength and temporal kinetics. Adapted from (Emiliani & Yizhar, 2022).

Channelrhodopsins (ChRs) are light-gated ion channels that allow ions to flow (mainly H^+ , Na^+ , but also K^+ , Cl^- and Ca^{++}) through the plasma membrane according to their electrochemical gradient (Deisseroth & Hegemann, 2017; Zhang et al., 2006). The first ChRs were discovered in a unicellular alga, *Chlamydomonas reinhardtii*, and were named CrChR1 (Nagel et al., 2002b) and CrChR2 (Nagel et al., 2003), later renamed ChR1 and ChR2 (Nagel

et al., 2002b, 2003). ChR2 was the opsin that ignited the development and use of optogenetics, due to its superior expression levels in mammalian cells (Adamantidis et al., 2007; Boyden et al., 2005; Deisseroth, 2015; Yizhar et al., 2011; Zhang et al., 2011). Still widely employed today to induce photoactivation in neurons, ChR2 is now only one of the many available options when selecting an optogenetic actuator (Boyden et al., 2005; Deisseroth, 2015; Deisseroth & Hegemann, 2017; Nagel et al., 2003; Zhang et al., 2006). Genomic mining (Zhang et al., 2008; Klapoetke et al., 2014; Marshel et al., 2019), and molecular engineerization of variants (e.g., Forli et al., 2021; Kishi et al., 2022; J. Y. Lin, 2011; J. Y. Lin et al., 2013; Mardinly et al., 2018; Printz et al., 2023; Vierock et al., 2021) have provided a multitude of cation- as well as anion-conducting channelrhodopsins that differ in ion permeability, spectral profile, light sensitivity, photo-current magnitude, and kinetics (Cosentino et al., 2015; Klapoetke et al., 2014; Madisen et al., 2012; Marshel et al., 2019; Mattis et al., 2011; Vierock et al., 2021, 2022; Wietek et al., 2014; F. Zhang et al., 2008; for review see Tan et al., 2022). Halorhodopsins (HRs) are electrogenic pumps responsible for chloride ions (Cl⁻) transportation inside the plasma membrane. As such, these opsins are inhibitory when expressed in neurons (Zhang et al., 2007). While early versions of these halorhodopsins (e.g., NpHR, F. Zhang et al., 2007; GtACRs, (Govorunova et al., 2015a) were characterized by membrane trafficking problems that led to variable, expression-level-dependent photocurrents, later developed variants have addressed this issue (Gradinaru et al., 2008, 2010; Mahn et al., 2018). For example, eNpHR3.0 (Gradinaru et al., 2008, 2010) is to date one of the most used inhibitory opsins in the community. Bacteriorhodopsins (BRs) are proton pumps responsible for the extrusion of protons (H⁺) from the cytoplasm to the extracellular space and are therefore used to silence neuronal activity (Deisseroth et al., 2015). Popular BRs are archaerhodopsin

(Arch; Chow et al., 2010) and its variants ArchT (higher light sensitivity; Han et al., 2011) and eArch3.0 (greater photocurrents; Mattis et al., 2011).

Effective delivery of opsins transgenes to the neuronal population of interest is crucial for efficient control of target neurons. One of the main factors determining maximal photocurrents in opsin-expressing neurons is the number of functional membrane-localized opsin molecules (Emilani & Yizhar, 2022). Several approaches are available to achieve robust expression in brain tissue: i) viral vector injections exploiting lentiviruses or adeno-associated viruses (AAV), possibly integrated with protein targeting motifs to spatially restrict opsin expression to cell bodies and controlling cell-type targeting via promoter selection (Lim et al., 2000; Wu et al., 2013); ii) transgenic animals constitutively expressing the opsin genes of interest; iii) transgenic animals expressing a recombinase system (e.g., Cre/lox; Nagy, 2000; Sternberg & Hamilton, 1981) either combined with the injection of a cre-dependent viral injection or crossed with a second transgenic animal strain carrying a floxed opsin gene sequence (e.g., (Bounds et al., 2023); iv) in-utero electroporation (IUE) of plasmids to target specific brain areas for opsin expression (Beltramo et al., 2013; Petreanu et al., 2007, 2009). Commonly, either AAV injections or transgenic lines are the most common strategies for opsins expression. However, both have limitations. AAVs require careful titration and validation. They can lead to inflammatory responses possibly affecting cell viability, have limited cargo capacity, can give rise to heterogeneous expression level across expressing neurons, and can potentially show tropism for neuronal subtypes different from the selected one (Alexander R. Nectow, 2020; Veres et al., 2023). Transgenic lines, instead, are mainly available in mice, whereas other animal models offer far fewer options. Moreover, transgenic animals can have a lower expression level compared to AAV injections (Dana et al., 2014; but see also Madisen et al.,

2012, 2015, Daigle et al., 2018, for contrasting evidence), although expression levels tend to be more homogeneous across cells in transgenic animals compared to AAV injections.

1.3.2 Optical approaches for optogenetic illumination

Neuronal circuits perturbation using optogenetics is mostly achieved with visible light delivery (< 700 nm) via 1-photon, wide-field illumination (1P optogenetics, Boyden et al., 2005; Yizhar et al., 2011; Y. P. Zhang & Oertner, 2007), employing optical fibres or miniaturized LEDs (e.g., Montgomery et al., 2015; Pisanello et al., 2014; Figure 7A, B). Due to the ease of implementation, many groups have successfully adopted 1P optogenetics (Kim et al., 2017). However, circuit dissection studies require spatially precise manipulation of neural activity (Figure 7). With 1P illumination, it is not possible to confine the excitation volume to single cells within the brain (Figure 7A). 1P optogenetics determines the simultaneous depolarization or hyperpolarization of cells within the illuminated volume, irrespective of their functional identity and contribution to the process under examination. Therefore, 1P optogenetics tends to generate non-physiological, hyper-synchronous responses among targeted neurons, which do not allow any conclusion on the importance of the precise spatiotemporal profile of network activity under normal circuit function, thus potentially leading to biased interpretation of results (Bovetti & Fellin, 2015; Carrillo-Reid et al., 2016; Emiliani et al., 2015; Emiliani & Ofer, 2022; Peron & Svoboda, 2011). Researchers have made several efforts to improve the spatial precision of 1P optogenetics, for example, using digital micromirror devices (DMDs; Guo et al., 2009; Leifer et al., 2011; Zhu et al., 2012) or computer-generated holography (CGH; Emiliani et al., 2015; Szabo et al., 2014) to shape the excitation volume by modulating either light intensity or light phase (Ronzitti et al., 2017); Figure 7C). However, when combined with

1P illumination, these methods failed to achieve single-cell resolution in densely labeled neural populations, especially in the axial direction (Emiliani et al., 2015). Two-photon excitation of opsins (2P optogenetics) overcomes these limitations. 2P optogenetics achieves near single-cell resolution and mitigates an often-overlooked issue in optogenetics studies: the unwanted depolarization of opsin-expressing neuronal processes and presynaptic terminals (Vaziri & Emiliani, 2012). Off-target stimulation or inhibition can bias results (Mahn et al., 2016, 2018; Rost et al., 2022), and a restricted illumination volume using 2P optogenetics can contribute to mitigate this problem (Wu et al., 2013). Moreover, to decrease off-target activation, experimenters coupled 2P optogenetics with soma-restricted opsin expression, which tends to lead to higher opsin expression levels at the soma compared to non-soma restricted approaches, resulting in more efficient activation of the opsin (Baker et al., 2016; Chettih & Harvey, 2019; Forli et al., 2018, 2021; Mahn et al., 2018; Mardinly et al., 2018; Shemesh et al., 2017).

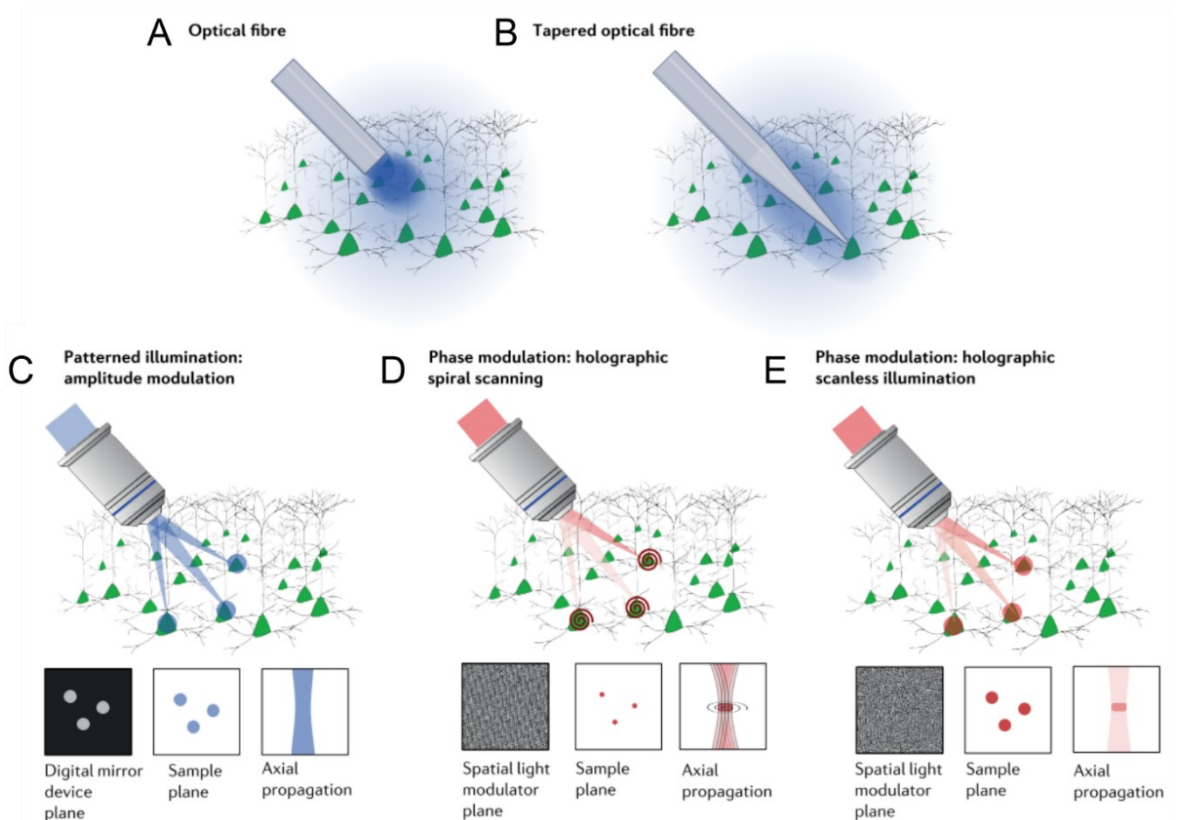


Figure 7. Optical approaches for 1P and 2P optogenetics.

A, B, C. 1P wide-field illumination of opsin-expressing neurons with flat-cleaved optical fibre (**A**), tapered fibre (**B**), and 1P patterned illumination of multiple targets with DMD-mediated beam intensity shaping (**C**). **D, E.** Two-photon illumination of opsin-expressing neurons by holographic light shaping with a spatial light modulator (SLM) that multiplexes the laser beam into: 3D distributed holographic spots, which are spirally scanned over targeted cell bodies (**D**); or extended shapes matching the cell body diameter and temporal focusing used to maintain micrometre axial resolution independently of lateral spot size (**E**). Adapted from (Emiliani & Yizhar, 2022).

Single-cell 2P optogenetics can be categorized into two main strategies: serial and parallel excitation (Figure 8). Serial excitation consists of raster or spiral scanning a high-power density spot with the galvanometer mirrors (Figure 7A, top). This strategy is usually employed in combination with opsins characterized by slow off kinetics and high conductance to temporally integrate photocurrents evoked at each scanned point (e.g., Carrillo-Reid et al., 2019; Chettih et al., 2019; Forli et al., 2021; Gill et al., 2020; Packer et al., 2015; Rickgauer et al., 2014; Yizhar et al., 2011a). Because of the integration time needed to drive opsin activation, the serial strategy usually does not guarantee high temporal fidelity (Bovetti & Fellin, 2015; Emiliani et al., 2015; but see Marshel et al., 2019). However, it has been shown that with longer illumination durations, serial excitation requires half the power of parallel approaches (Yang et al., 2018), optimizing the laser power budget and therefore increasing the number of neurons that can be targeted simultaneously. Taken together, these characteristics make serial excitation a valuable tool for population-level experiments, where the focus is on the identity and number of activated neurons rather than the precise timing of evoked spikes. If the main goal is instead to achieve temporally precise optogenetic activation of neurons, parallel excitation coupled with fast opsins (e.g., Chronos, Klapoetke et al., 2014; Ronzitti et al., 2017, or ChroME, Mardinly et al., 2018; Sridharan et al., 2022; Shemesh et al., 2017) should be preferred. Parallel

approaches rely on light-sculpting techniques (computer-generated holography, generalized phase contrast, temporal focusing) to generate an extended shape over the cell body of target neurons, leading to simultaneous excitation of a large number of opsins and inducing larger instantaneous photocurrents compared to serial scanning.

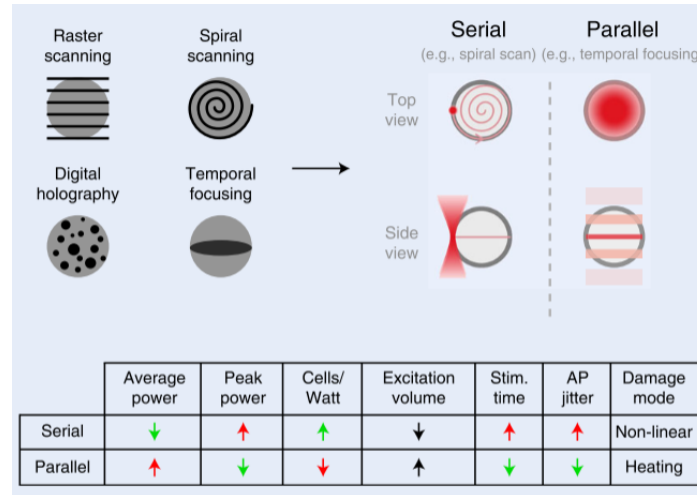


Figure 8. Serial and parallel illumination methods

Serial illumination strategies are more suitable for experiments prioritizing the number of simultaneously targeted neurons and employment of large-conductance opsins. Parallel excitation strategies are more suited to experiments requiring temporal fidelity of AP generation, or for dense multi-site patterns for which fast scanning is not possible. Favorable (green) and unfavorable (red) properties of each method are indicated. Grey circles represent cross sections of cell somata. Adapted from (Russell et al., 2022).

Both excitation strategies can cause tissue damage. Given their small, high-density excitation volume, serial methods are prone to causing non-linear damage (Hopt & Neher, 2001; Koester et al., 1999). Because of the higher average power delivered to cells, parallel excitation methods are instead more likely to generate heating damage (Picot et al., 2018). Multiple other factors contribute to determining both types of damage: average power delivered, stimulation duration, laser repetition rate (Hopt & Neher, 2001; Koester et al., 1999; Podgorski & Ranganathan, 2016). For thermal damage, the experimenter should also consider spatial

distribution, number, and inter-distance of excitation sites (Picot et al., 2018). More in general, minimizing stimulation duration and average power should always be prioritized in all-optical experiments (Russel et al., 2022), and experiment-specific calibration should ideally be carried out for all parameters.

1.3.3 Light sculpting for simultaneous illumination of multiple neurons

Patterned illumination approaches generate complex light distributions via intensity or phase modulation (Bovetti & Fellin, 2015; Dal Maschio et al., 2012; Papagiakoumou et al., 2008, 2009; Ronzitti et al., 2017; Vaziri & Emiliani, 2012). Computer-generated holography (Emiliani et al., 2015; Lohmann & Paris, 1967) or the generalized phase contrast (GPC; Bègue et al., 2013; Bovetti & Fellin, 2015; Glückstad, 1996; Mogensen & Glückstad, 2001; Papagiakoumou et al., 2010; Ronzitti et al., 2017) methods, mostly implemented with liquid crystal spatial light modulators (LC-SLM), can be exploited to generate parallel excitation of single cells (Bañas & Glückstad, 2017; Glückstad & Palima, 2008; Packer et al., 2013). CGH and GPC are also employed to multiplex the laser beam into multiple beamlets, which can either be focal spots – an “hybrid approach” combining serial excitation and holographic multiplexing (Packer et al., 2012, 2015; Yang et al., 2018) or multiple extended shapes into profiles that cover single cell somata (Dal Maschio et al., 2010; Papagiakoumou et al., 2010). Patterned illumination approaches therefore allow true simultaneous and efficient illumination of multiple neurons (Bovetti & Fellin, 2015; Dal Maschio et al., 2010; Ronzitti et al., 2017). SLMs modulate either the phase (Bovetti & Fellin, 2015; Dal Maschio et al., 2010; Ronzitti et al., 2017; Yang et al., 2023) or the intensity (Guo et al., 2009; Leifer et al., 2011; Zhu et al., 2012) of the incident beam. A liquid crystal spatial light modulator is a device that enables

modulation of light by means of a 2D matrix of active pixels, each enclosing liquid crystals (LC, Figure 9B). Liquid crystals are polar and birefringent molecules. Therefore, within each pixel LCs can be differentially oriented with respect to the direction of propagation of light by imposing an electric field on the dielectric medium they are immersed in (Figure 9B). LC reorientation changes the effective refractive index that light undergoes while traveling in the medium where the LCs are immersed, generating a local modulation of light diffraction properties within each matrix pixel (Bovetti & Fellin, 2015; Dal Maschio et al., 2010, 2012). By appropriate coordination of the voltage difference applied to the whole set of pixels in the 2D matrix it is possible to perform efficient and dynamic phase modulation of an impinging planar laser wave-front (Figure 9C). In the case of reflective LC SLMs, the resulting complex wave-front is then reflected and refracted away from the SLM and directed to the subsequent optical components of the optical path. This method is frequently called wave-front shaping, wave-front engineering, or computer-generated holography (CGH; Di Leonardo et al., 2007; Gerchberg, 1972; Sinclair et al., 2004; Wyrowski & Bryngdahl, 1991).

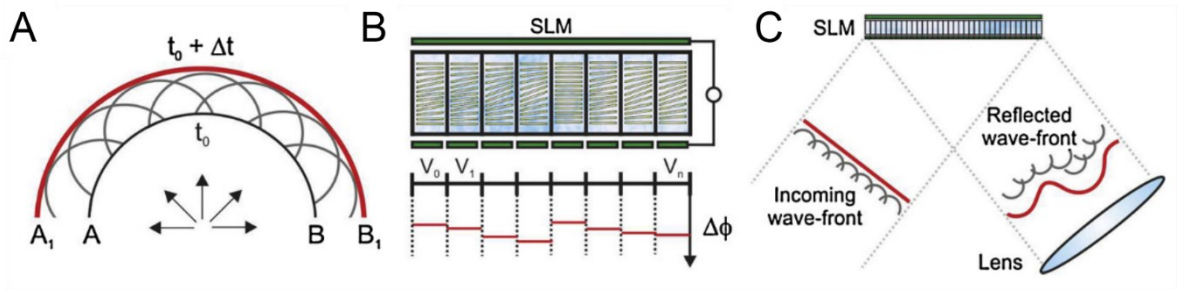


Figure 9. Liquid crystal spatial light modulators

A. Schematic representation of the Huygens–Fresnel principle. **B.** Each pixel cell of the LC-SLM contains liquid crystals immersed in a dielectric medium (top). A voltage difference applied to individual pixel electrodes (green rectangles) changes the orientation of the liquid crystals, modifying the pixel’s refractive index and generating different phase delays (bottom). **C.** The planar wave-front of a laser beam is transformed into a complex wave-front by phase modulation with the LC-SLM. Adapted from (Bovetti and Fellin, 2015).

In the approach described above, phase modulation is performed in a plane optically conjugated with the back focal plane of the objective (BFP, the Fourier plane), leading to modulation of light intensity at the sample plane (i.e., frontal focal plane, FFP) of the objective (Bovetti & Fellin, 2015; Ronzitti et al., 2017). The pattern of voltages to be applied to the matrix of LC SLM pixels correspond to a pattern of phase delays (also called phase map) applied to the reflected wave-front. Phase maps are typically computed by applying iterative algorithms (from which the term computer-generated holography was derived (Bañas & Glückstad, 2017)).

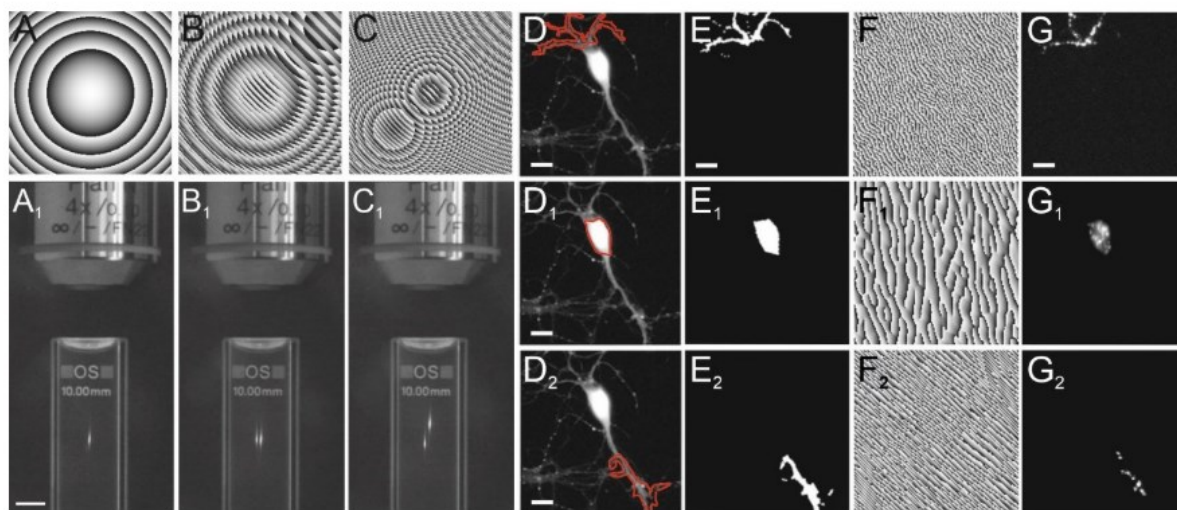


Figure 10. Patterned illumination with liquid crystal spatial light modulators

A – C. Examples of phase maps. **A1 – C1.** A cuvette of fluorescein is excited by two-photon illumination spots corresponding to the phase maps shown in (A – C). The two spots are generated on the same plane (**B1**) or on two distinct planes (**C1**). $\lambda = 800$ nm; scale bar, 5 mm. **D – D2.** Fluorescence image showing a cultured neuron filled with Fluo-4. The regions of interest (red line) corresponding to the cell body (**D1**), or portion of the dendritic tree (**D** and **D2**) are shown. Scale bar: 10 μ m. **E – E2.** A binary mask corresponding to the selected regions of interest is generated: a portion of the dendritic tree in (**E**) and (**E2**), or of the cell somata (**E1**). **F – F2.** Phase maps corresponding to the binary masks shown in (E – E2). **G – G2.** Fluorescence images showing patterned two-photon illumination in the regions of interest displayed in (D – D2). Adapted from (Bovetti & Fellin, 2015).

One limitation of CGH is the introduction of inhomogeneities (“speckles”; Figure 10G – G1 – G2) in the generated intensity profile caused by limitations of the applied algorithms and pseudorandom constructive or deconstructive interferences from surrounding light rays (Dal Maschio et al., 2012; Sinclair et al., 2004). Both hardware and software solutions have been developed to address spatial inhomogeneities (Dal Maschio et al., 2012; Vaziri & Emiliani, 2012; Bovetti & Fellin, 2015; Ronzitti et al., 2017a). One “hardware” solution is generalized phase contrast (GPC), which optically converts user-defined phase variations into proportional amplitude variations at the sample plane (GPC; Zernike, 1955; Glückstad et al., 1996; Papagiakoumou et al., 2010; Bègue et al., 2013; Bovetti & Fellin, 2015; Ronzitti et al., 2017a). One “software” solution is the use of the weighted Gerchberg-Saxton (WGS) iterative algorithm (Di Leonardo et al., 2007). Although computationally expensive, this inverse-Fourier transform procedure can compensate for power losses at the extremes of the addressable area of the field of view (Dal Maschio et al., 2017; Russell et al., 2022). After its first successful application for 2P optogenetics (Andrasfalvy et al., 2010; Mohanty et al., 2008; Papagiakoumou et al., 2010; Rickgauer et al., 2014), CGH has become a relatively common method to perform photostimulation both *in vitro* and *in vivo*, with extended shapes or by multiplexing single diffraction-limited spots.

1.4 Optimization of simultaneous two-photon imaging and optogenetics: “all-optical” experiments

The first two-photon all-optical experiments combined calcium imaging and glutamate uncaging *in vitro* (Anselmi et al., 2011; Dal Maschio et al., 2010; Lutz et al., 2008; Nikolenko et al., 2007). In parallel, different research groups demonstrated 2P activation of

channelrhodopsin-2 (Mohanty et al., 2008; Papagiakoumou et al., 2010; Rickgauer & Tank, 2009) and other opsins (Yizhar et al., 2011; Prakash et al., 2012). In 2014, Rickgauer and colleagues (Rickgauer & Tank, 2009) reported the first simultaneous 2P imaging and photostimulation experiment in awake behaving mice. The authors recorded hippocampal neurons with the transgenically expressed calcium reporter GCaMP3 ($\lambda_{\text{imaging}} = 920 \text{ nm}$) and sequentially manipulated the activity of excitatory neurons co-expressing the red-shifted opsin C1V1 ($\lambda_{\text{stim}} = 1064 \text{ nm}$) while mice navigated a virtual environment (Rickgauer & Tank, 2009). One year later, Packer et al. described 2P all-optical experiments in awake behaving animals using a similar indicator-opsin strategy (co-injection of GCaMP6s and C1V1, same wavelengths as in Rickgauer et al., 2014), but they achieved patterned optogenetic excitation of multiple neurons by using the SLM to multiplex the laser beam into multiple diffraction-limited spots, which were then spiral scanned over the target neuronal cell bodies. Since these earlier studies, it was clear that two-photon all-optical experiments enabling simultaneous monitoring and perturbation of neuronal networks with near cellular resolution are an extremely powerful tool for the investigation of the relationship between specific patterns of neuronal activity within a given circuit and behavior. However, these same earlier studies also pointed out to the limitations of this approach. Specifically, both Rickgauer et al. (2014) and Packer et al. (2015) reported the presence of potential optical crosstalk in their studies. Optical crosstalk refers to two different phenomena: i) an illumination artifact present during the acquisition of fluorescence images during the delivery of photostimulation light (Akerboom et al., 2013; Packer et al., 2015; Forli et al., 2018); and ii) the unwanted activation of the opsin by the laser beam used to image the fluorescent functional indicator (Packer et al., 2015; Forli et al., 2018; Figure 11). Both types of crosstalk are due to the partial overlap between the

absorption spectra of fluorophores (including functional indicators and fluorescent tags attached to the opsin) and opsins used in two-photon all-optical experiments. The first type of crosstalk usually generates artefactual values of fluorescence intensity during scanning. Since photostimulation time is usually shorter than the time required for image acquisition, this type of artifact can be mitigated by synchronizing photostimulation events with the scanning system to minimize its impact on image acquisition (e.g., Yang et al., 2018).

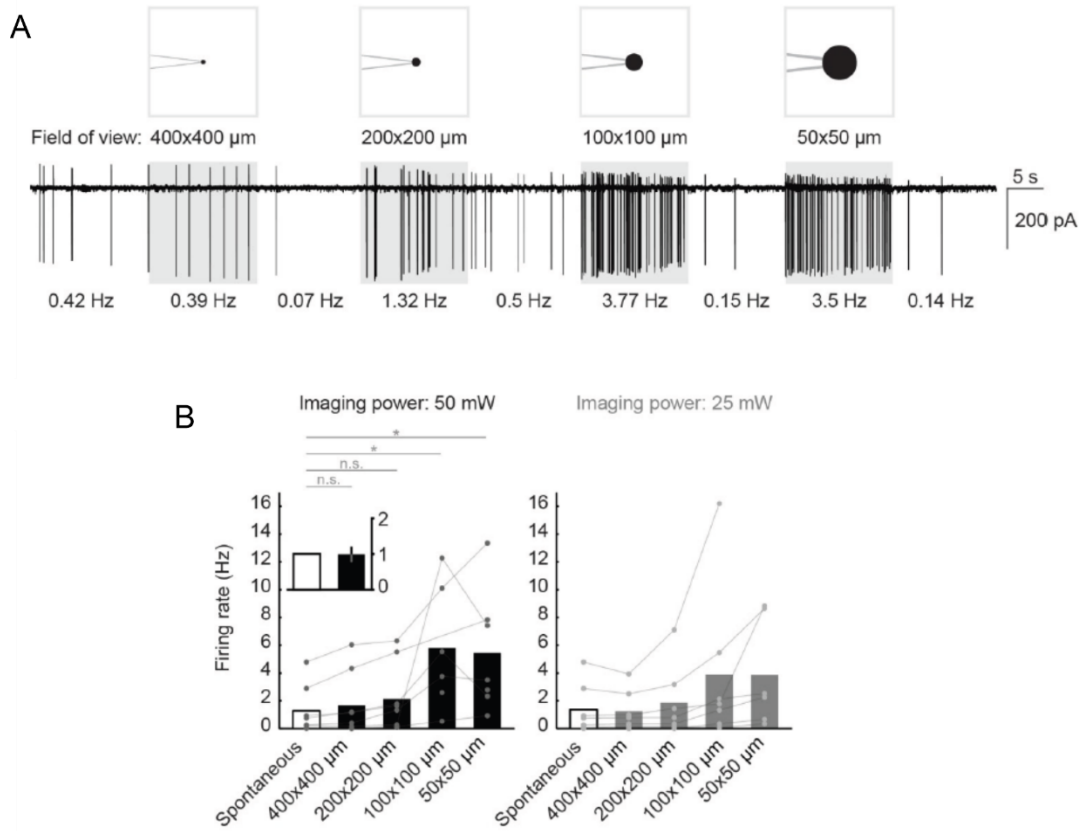


Figure 11. Crosstalk between imaging and photostimulation: increasing optical zoom during imaging leads to neuronal depolarization

A. Representative cell-attached patch-clamp recording from one neuron. Imaging periods are indicated by the shaded regions. Imaging was performed at 30 Hz with 920 nm excitation while the size of the field of view (optical zoom) was changed. Decreasing the FOV size increased AP probability. **B.** Quantification of (A) for 2 different imaging powers, 50 mW and 25 mW average power. Inset shows firing rates while imaging a 400x400 μm field of view, normalized to the recorded cells spontaneous firing rate. From Packer et al. 2014).

For the second type of crosstalk (between imaging and photostimulation), it is important to underline that virtually all known opsins display a blue-shifted tail in their absorption spectra (Kishi et al., 2022; Marshel et al., 2019; Sridharan et al., 2022; Zhang et al., 2011). This spectral overlap determines the optical crosstalk. This type of crosstalk worsens with large-conductance, slow off-kinetics opsins (e.g., C1V1, Chrimson, and ChRmine), which are commonly used in combination with a green calcium indicator (e.g., GCaMPs). Crosstalk between imaging and photostimulation can be mitigated by lowering imaging laser power (Figure 11B, right), decreasing the acquisition frame rate, or increasing the field of view dimensions to minimize the laser dwell time over individual cells (Marshel et al., 2019; Mardinly et al., 2018; Packer et al., 2015; Sridharan et al., 2021; Figure 10A). However, these strategies affect the SNR of fluorescence signals, the temporal resolution of the recordings, or both. An alternative strategy to minimize crosstalk between imaging and photostimulation is the use of blue-shifted opsins with red-shifted calcium indicators to minimize the spectral overlap between the absorption spectra of the two molecules (Forli et al., 2018; Forli et al., 2021; Yizhar et al., 2011a; Venkatachalam & Cohen, 2014). In Forli et al. 2018, the authors co-expressed the red calcium indicator jRCaMP1a (Dana et al., 2016) and the blue-shifted opsin ChR2 (Boyden et al., 2005; Nagel et al., 2003), demonstrating limited activation of ChR2 and its soma targeted version stChR2 during imaging periods at 1100 nm and effective activation of neurons targeted with extended shapes at 920 nm. It is important to underline that the enrichment of opsin expression within the somatic and perisomatic compartments has been demonstrated to significantly enhance photocurrent amplitude and refine the spatial resolution of photostimulation, reducing the off-target stimulation probability that may occur with non-soma-restricted opsins (Baker et al., 2016; Chettih & Harvey, 2019; Forli et al., 2018; Mardinly

et al., 2018; Marshel et al., 2019; Shemesh et al., 2017). However, ChR2 has low conductance, inducing limited change in neuronal spiking activity upon laser illumination (Forli et al., 2018). Moreover, the jRCaMP1a indicator has limited sensitivity, brightness, and SNR compared to commonly employed green-emitting indicators, such as GCaMPs. To address the limitation linked to the use of ChR2, a new soma-targeted variant (st) of the blue-light-sensitive, large-conductance opsin CoChR (Klapoetke et al., 2014) was introduced in all-optical experiments (Forli et al., 2021; Figure 12). The use of stCoChR enabled one order of magnitude larger neuronal responses with respect to using stChR2 (Forli et al., 2021). Moreover, photostimulation of neurons with a high energy, low repetition rate laser and an optical parametric amplifier (~4.5 W at 920 nm, 1 MHz, Forli et al. 2021) rather than low energy, high repetition rate (~1.8 W at 920 nm, 80 MHz, Forli et al 2018) allowed to decrease the average power delivered to individual cells to 0.5-5 mW per cell (see also Yang et al., 2018), thus reducing issues related to potential thermal effect of photostimulation on the brain tissue (see section 1.3.2) and enabling larger number of neurons to be stimulated.

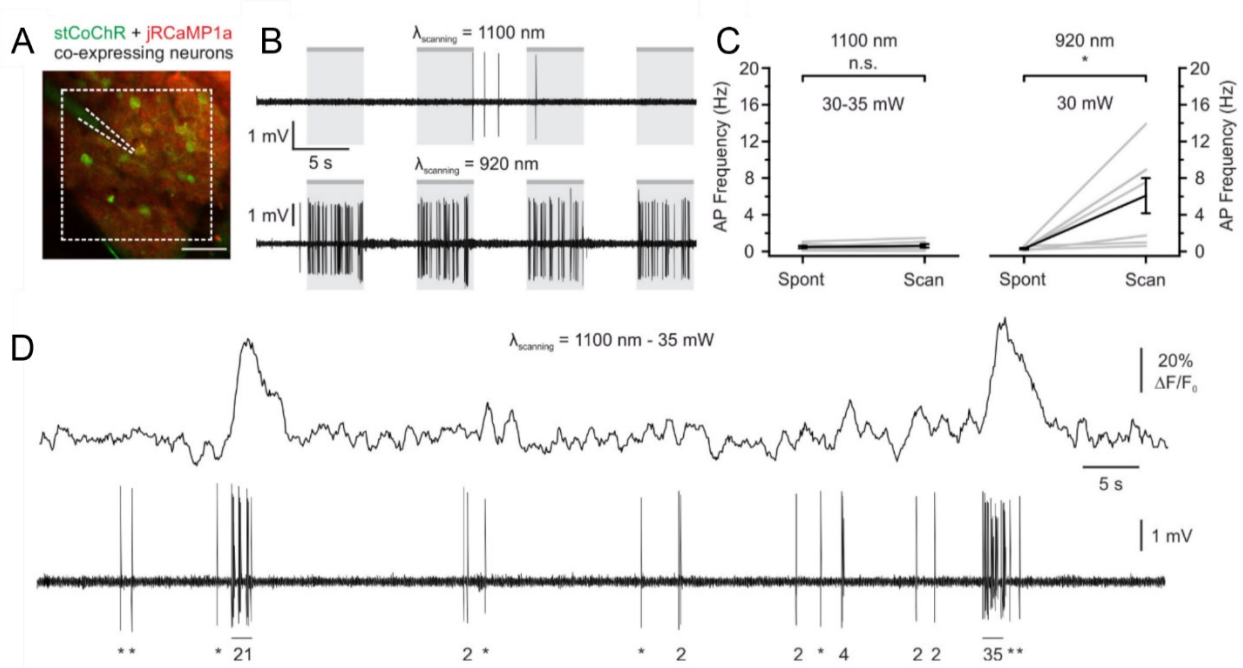


Figure 12. Limited crosstalk between imaging and photostimulation using blue-light-sensitive opsins stCoChR and red-shifted indicator jRCaMP1a

A. Two-photon image of L2/3 pyramidal cells co-expressing stCoChR and jRCaMP1a *in vivo*. Cell-attached recordings of single neurons while two-photon raster scanning inside the indicated area (dashed white box, 161.4 x 161.4 μm^2 ; imaging frame rate: 11 Hz) were performed; **B.** Recorded traces from one co-expressing neuron during two-photon raster scanning epochs (grey boxes) at wavelength 1100 nm (top) and 920 nm (bottom). Laser power: 30 mW; **C.** Average AP frequency during epochs of spontaneous activity (Spont) and during raster scanning (Scan). Left: results when scanning was performed at $\lambda = 1100$ nm. Right: results when scanning was done at $\lambda = 920$ nm. $n = 11$ cells from six mice. **D.** Representative trace from a L2/3 pyramidal neuron co-expressing stCoChR and jRCaMP1a. AP firing was recorded in the juxtасomal configuration (bottom) while two-photon raster scanning was performed (top). Imaging frame rate and FOV dimensions as in (A); average imaging power, 35 mW. The number of recorded APs is reported below the electrophysiological trace (single APs are indicated with an asterisk). Adapted from (Forli et al., 2021).

The approach proposed by Forli et al. (2021) still relies on the red-shifted indicator jRCaMP1a, which lacks the SNR, kinetics, and dynamic range of green-shifted indicators. Newly engineered red-shifted indicators have been recently developed (Yokoyama et al., 2024; Fink et al., Dalangin et al., 2025; see section 1.2.2), holding promising perspectives to improve all optical experiments with reduced crosstalk between imaging and stimulation.

Irrespective of the indicator and opsin of choice, all-optical experiments frequently rely on the injection of two separate viruses to infect selected neuronal populations (Nectow & Nestler, 2020). Such strategies may lead to heterogeneous ratios between the expression levels of the two proteins in the same cells (Carrillo-Reid et al., 2019; Chettih & Harvey, 2019; Gill et al., 2020; Packer et al., 2015) and in some cases to segregation of expression of the opsin and indicator in different cells (LaFosse et al., 2023; Packer et al., 2015; Russell et al., 2022). Optimization of the viral co-expression protocol achieves an appropriate and balanced co-expression ratio between the functional indicator and the opsin, thereby maximizing the

number of available cells that co-express the opsin and the functional indicator (Dalglish et al., 2020; Marshel et al., 2019; Packer et al., 2015; Russell et al., 2022). Alternative strategies to the co-expression of the opsin and the indicator include the use of a bicistronic construct inserted in a single virus and transgenic animals constitutively expressing both proteins. Bicistronic constructs have been developed for red-shifted opsins and green-shifted indicators. For example, Marshel et al. (2019) proposed a single bicistronic virus encoding a soma-restricted version of the red-shifted opsin ChRmine and the green calcium indicator GCaMP6m. They reported strong and stable expression in layer 2/3 and layer 5 neurons and no significant activation of ChRmine-expressing cells in their experimental conditions. However, ChRmine was later shown to be photoactivated by 920 nm light exposure (Sridharan et al., 2022), leading to significant crosstalk between imaging and photostimulation. Another bicistronic construct encoding a green indicator and a red opsin was developed a few years later (LaFosse et al., 2023). This second bicistronic construct expressed jGCaMP8s and stChrimsonR (Sridharan et al., 2022). They reported no significant signs of optical crosstalk. However, their assessment was based on average cellular activity measured via 2P calcium imaging, which may miss individual spikes (Dana et al., 2014, 2016). A more conclusive evaluation would require electrophysiological data to rule out moderate supra-subthreshold activation of neurons upon 920 nm illumination. Finally, the only construct carrying a red calcium indicator and a blue-shifted opsin to date combines PinkyCaMP and stCoChR (Fink et al., 2023; see section 1.2.2). However, this construct was only used in combination with 1P illumination and the 2P all-optical experiments remains to be tested.

Regarding transgenic mouse lines suitable for 2P all-optical experiments, only one line has been created to date (Bounds et al., 2023). This transgenic line (called Ai203) uses the

TIGRE2.0 approach to enable Cre-dependent co-expression of the ultra-fast opsin ChroME and the calcium indicator GCaMP7s, fused into a single soma-targeted protein (Bounds et al., 2023). In vivo, the line exhibited robust, soma-restricted expression, supports high-fidelity two-photon optogenetic stimulation, and enables sensitive calcium imaging with minimal crosstalk between imaging and stimulation wavelengths (Bounds et al., 2023). Loose-patch recordings in anesthetized mice did not show statistically significant changes in normalized AP frequency, even at high imaging powers (> 60 mW). However, this data was acquired by imaging a large field of view ($680\text{ }\mu\text{m} \times 680\text{ }\mu\text{m}$) in anesthetized animals (i.e., when cells tend to have a more depolarized membrane potential with respect to non-anesthetized conditions).

1.5 Probing the contribution of neural activity patterns to behavior

The 2P all-optical approach described in the previous sections has opened up the possibility of addressing previously unattainable questions in experimental neuroscience. One prime example relates to the fact that 2P all-optical approaches allow for the first time to directly test the functional role of co-active groups of neurons (also termed ensembles, cell assemblies, motifs, see Yuste et al., 2024 for a comprehensive review on the topic and related terminology) in behavior (Buetfering et al., 2022; Carrillo-Reid et al., 2019; Daie et al., 2021; Dal Maschio et al., 2017; Dagleish et al., 2020; Gill et al., 2020; Packer et al., 2015; Rickgauer & Tank, 2009; Russell et al., 2024). While the hypothesis that ensembles of neurons constitute the emerging functional units of brain function and behavior has a long history in neuroscience (Abeles, 1991; Buzsáki, 2010; Hebb, 1949; Hopfield, 1982; Rosa Cossart et al., 2003; Sherrington, 1906), technical limitations have long prevented researchers from directly testing

it by simultaneously recording and perturbing specific subpopulations of neurons in behaving animals. Three seminal papers addressed this hypothesis.

Dal Maschio et al. (2017) first demonstrated the use of all-optical experiments in vivo to functionally dissect neuronal circuits implicated in behavior. In this study, the authors investigated the nucleus of the medial longitudinal fascicle (nMLF) of the zebrafish midbrain, a structure known to be implicated in tail posture and locomotor control (Thiele et al., 2014). The optical setup enabled volumetric two-photon calcium imaging (via an electro-tunable lens, ETL) and 2P holographic photostimulation. Transgenic larval zebrafishes co-expressing ChR2 and either GCaMP6s or CaMP5G were employed. As this combination of opsin and indicator was acknowledged to generate optical crosstalk, the imaging wavelength was set at 1020 nm, while photostimulation was delivered at 920 nm, resulting in minimized ChR2 activation during imaging. A high-speed camera was used to record the motor behavior of interest (tail bending) and relate it to the neuronal signal. Photostimulation was first confirmed to elicit detectable calcium transients. In the following experiment, the authors applied bulk stimulation to nMLF neurons, and then progressively restricted it to smaller ensembles until the minimal number of neurons causally related to tail movement and swimming was identified. An independent component analysis (ICA) was subsequently applied to extract activity components most predictive of behavior. The identified components were then integrated into a regularized linear regression model to estimate tail angle from recorded activity. Leveraging a 550 μm wide field-of-view, it was possible to analyze activity in the larval midbrain and, also, hindbrain. This examination revealed focal “hotspots” of activity in the hindbrain temporally aligned with photostimulation -and behavioral- events, as well as cells exhibiting out-of-phase activity. Finally, in animals co-expressing photoactivatable GFP (paGFP)

together with ChR2 and GCaMP the authors demonstrated the possibility to exploit paGFP photoconversion to morphologically characterize functionally defined neurons (Dal Maschio et al., 2017).

Second, Carrillo-Reid and colleagues in 2019 (Carrillo-Reid et al., 2019) trained animals co-expressing GCaMP6s and C1V1 in layer 2/3 pyramidal neurons of the primary visual cortex (V1) to lick from a waterspout at the occurrence of the visual “Go” stimulus and refrain from licking with the “No-Go” stimulus. With 2P imaging, the authors identified specific subgroups of neurons in the imaged field of view that were reliably activated in correspondence with the “Go” stimulus (Go ensembles) or the “No-Go” stimulus (No-Go ensembles). Also, they reported the presence of “pattern completion” neurons, i.e., neurons computationally classified as having the strongest functional influence on the Go or No-Go ensemble they belonged to. The investigators performed three sets of experiments to investigate the causal role of these ensembles in the behavioral task. First, they optogenetically activated the Go and No-Go ensembles during the corresponding Go and No-Go trials, resulting in improved performance. Second, they repeated the same experiment but only activated pattern completion neurons: direct stimulation of pattern completion cells recalled the whole Go or No-Go ensemble (depending on whether the pattern completions cells belonged to the Go ensemble or to the No-Go ensemble), also leading to improved performance. Third, the optogenetic activation of the pattern completion neurons belonging to the Go ensembles in the absence of visual stimulation resulted in increased licking responses consistent with the perception of the virtual Go stimulus. Finally, in a separate set of experiments the authors tested the impact of randomly photoactivating neurons during the presentation of the Go stimulus. Random neuronal stimulation test resulted in a decrease in performance, suggesting that uncoordinated neuronal

activation leads to the disruption of the functional effects of behavior-related ensembles (Carillo-Reid et al., 2019).

In the third seminal paper, Marshel et al. (2019) investigated how distinct visual stimuli are represented in the mouse primary visual cortex (V1) during a visual discrimination task and how the number and coordination of responsive neurons generate the perception of the visual stimulus. They co-expressed a newly engineered red-shifted opsin (ChRmine) and GCaMP6m in both layer 2/3 and layer 5 principal cells via the injection of a bicistronic viral vector encoding both protein-coding sequences. The authors also implemented an all-optical approach that enabled fast scanning (up to 1 kHz) of multiple focal spots on targeted neurons through a custom dual-SLM system. Naïve mice were first imaged while vertical (0°) and horizontal (90°) gratings were presented to allow the identification of stimulus-tuned ensembles. Non-overlapping tuned ensembles comparable in size were identified for both stimuli across layers. A third class of two control ensembles, each of matching size, was selected from the surrounding neuronal population, paired with each of the two visually tuned ensembles. Trials of visual or optogenetic 2P holographic stimulation of each of the three ensemble classes were interleaved. Mice were then trained to discriminate between the two visual cues and report the Go (0°) stimulus by licking while withholding for the No-Go (90°) stimulus, even as stimulus contrast was progressively reduced. The lowest contrast (2%) visual stimuli were insufficient to reach higher than chance performance. Importantly, coupling low contrast visual stimuli with optogenetic activation of the appropriate neuronal ensemble re-enabled correct discrimination of the two stimuli. Further analysis revealed that, throughout training, stimulus-tuned ensembles were able to recruit neurons from the surrounding network with similar tuning properties, rather than those with opposite tuning properties. This suggested that selective

recruitment could be operating during learning. Ultimately, by performing a combined analysis on photostimulated and naturally stimulus-responsive neurons, the authors were able to elaborate on the connectivity patterns within and across layers 2/3 and 5, describing a circuit-level model that accounts for the observed behavioral outcomes (Marshall et al., 2019).

A series of subsequent studies investigated the link between patterned illumination at the circuit level and behavior (Bounds & Adesnik, 2025; Buetfering et al., 2022; Dalgleish et al., 2020; Gill et al., 2020; Piantadosi et al., 2024; Robinson et al., 2020; Russell et al., 2024; Shin et al., 2023), mostly focusing on sensory perception. For example, Russell et al. (2024) trained mice in a visual stimulus detection task (drifting gratings of varying contrasts) to investigate the influence of animals' behavioral state on sensory perception. To do so, the pupil diameter of the animals, a widely recognized proxy of cholinergic activity and arousal state of animals (Joshi et al., 2016, 2020; Reimer et al., 2016) was monitored during the experimental session. Coupling pupil size measures with a parameter evaluating neuronal synchronous activation (Figure 13 A, B), the authors were able to identify multiple behavioral states characterized by more- or less engagement (Russell et al., 2024). Researchers then used volumetric 2P calcium imaging and optogenetics to photostimulate neurons in layer 2/3 of V1 co-expressing GCaMP6s and C1V1 tuned to the visual stimulus. Similarly to what was reported by Carrillo-Reid and colleagues, an enhancement of low-contrast stimuli detection when co-tuned neurons were optogenetically targeted during the presentation of the stimulus was observed. However, sensory perception enhancement occurred specifically in low-, but not high-contrast stimuli, during the behavioral states characterized by higher engagement (Russell et al., 2024). An opposite, suppressive effect was observed in the high-contrast, more engaged state condition. In the less engaged state, photostimulation did not alter behavior. Interestingly, the authors

reported that “while not an integral part of the task design, we found that allowing mice to be free to run improved their performance in the task” (Russell et al., 2024). How running activity influences sensory perception and whether the optogenetic activation of neurons changes during running epochs remains, therefore, still to be clarified.

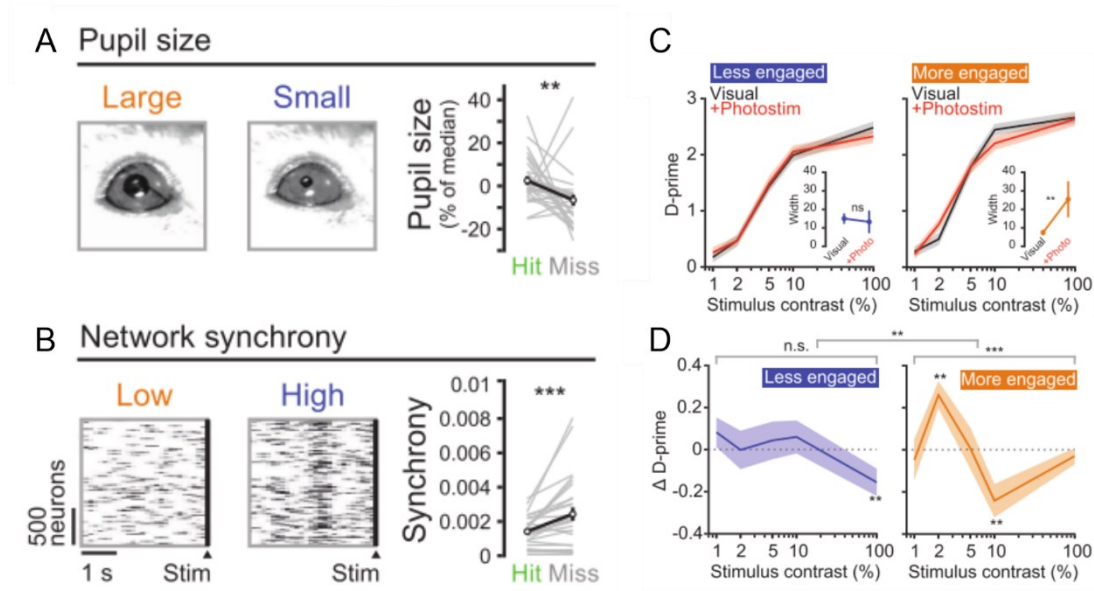


Figure 13. Level of engagement measures and targeted photostimulation of stimulus coding neurons in L2/3 neurons of the mouse V1

A. Pupillometry: large and small pupil sizes reflect different behavioral states; **B.** Network synchrony is computed as the average pairwise correlation between the signals extracted from deconvolved activity traces of all pairs of cells in the pre-trial period. Low and high synchrony reflect different behavioral states; **C.** Photostimulation of co-tuned ensembles paired with visual stimuli. Sessions are split into two states, ‘less engaged’ and ‘more engaged’. Black lines: performance on visual-only trials. Red lines: performance on visual + photostimulation trials. Inset: width of fitted psychometric curve; **D.** Photostimulation has a consistent effect on the detectability of visual stimuli in the ‘more engaged’ state and not in the ‘less engaged’ state. Photostimulation enhanced detection of low (2%) contrast stimuli and suppressed the detection of higher (10%) contrast stimuli.; in **(D)** significance across each full curve. Adapted from (Russell et al., 2024).

Overall, the findings from Russell et al. corroborate a causal role of neuronal ensembles in sensory perception. While what a neural ensemble actually encodes and what its role is within the meso- and macro-scale level of network activity remains an open question (Buzsáki, 2010),

all-optical studies provide causal evidence for the role of groups of co-active neurons in influencing specific aspects of animal behavior, and start opening the way to the investigation of what spatial and temporal feature of the activity patterns influence their functional roles (Dalglish et al., 2020; Furutachi et al., 2024; Robinson et al., 2020). Tackling these questions requires continuous development of 2P all-optical approaches. Volumetric all-optical 2P experiments, for example, yield important insights into the cross-layer interactions occurring during behaviors (Ayaz et al., 2019; Marshel et al., 2019; Ronzitti et al., 2018) or expand the number of available neurons for imaging and photostimulation within a cortical layer (Pégaré et al., 2017; Yang et al., 2018). Similarly, mesoscopic 2P all-optical approaches provide a unique window to investigate how activity patterns across functionally distinct cortical regions influence behavior (Abdeladim et al., 2023) (Figure 14). Furthermore, connectivity mapping studies can also be effectively carried out in vivo and during behavior with 2P all-optical experiments (Chen et al., 2025, 2016; Chettih & Harvey, 2019; Fişek et al., 2023; Khoury et al., 2025; Oldenburg et al., 2024; Printz et al., 2023; Spampinato et al., 2022; Triplett et al., 2025; Vinograd et al., 2024). At present, connectivity studies are technically hindered by the slow dynamics of calcium indicators, making them best suited for cross-area mapping investigations (e.g., Chen et al., 2016; Fişek et al., 2023; Printz et al., 2023). The advent of new and better performing GEVIs (Carolan et al., 2025; Grewe et al., 2010; Hao et al., 2024; Villette et al., 2019) and high-speed scanning approaches (Duemani Reddy et al., 2008; Grewe et al., 2010; Saggau et al., 2019; Villette et al., 2019; Zhong et al., 2025), however, will likely improve the temporal resolution of all optical 2P experiments. 2P all-optical experiments will also benefit from closed-loop approaches, allowing precise tailoring of intensity and timing of photostimulation depending on online recorded activity. Zhang and colleagues (2018)

pioneered this approach and demonstrated, for example, optical clamping of neuronal activity to an arbitrary threshold and patterned photostimulation of neurons triggered by the activity of identified cells within the FOV.

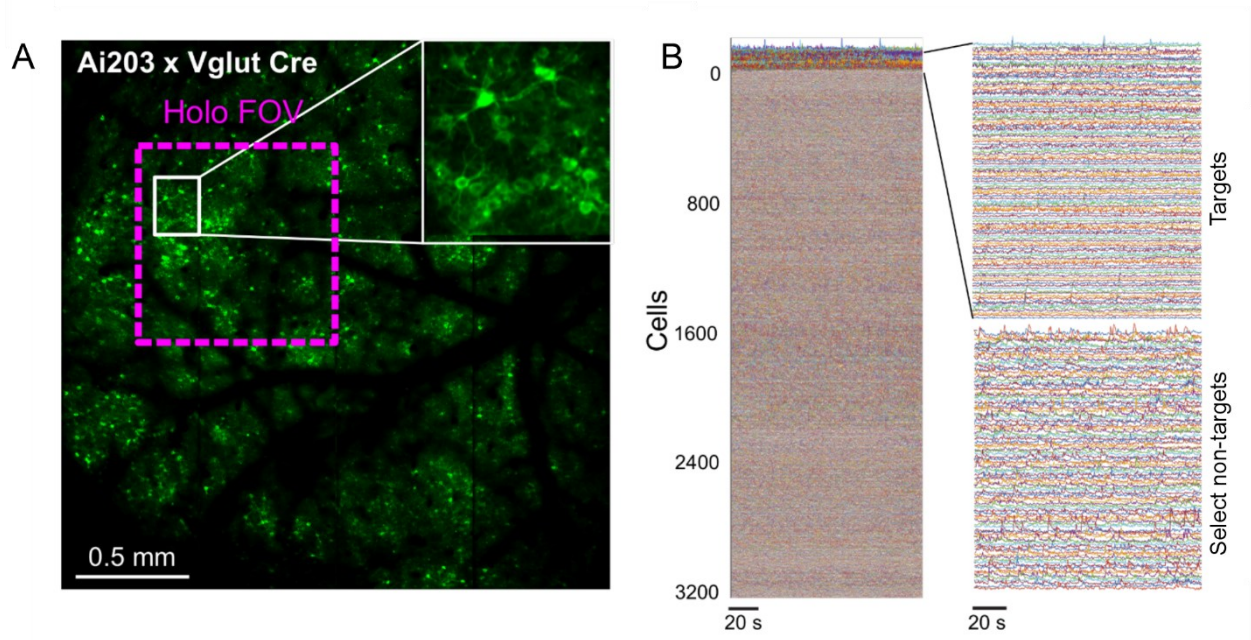


Figure 14. Mesoscopic two-photon holographic optogenetics.

A. Two-photon mesoscale image of neurons co-expressing GCaMP7s and ChroME over a large field-of-view (2.4 mm × 2.4 mm recorded at 4.5Hz) in a Vglut1-Cre/Ai203 transgenic mouse. Accessible holographic FOV is indicated in magenta; **B.** Example calcium traces from photostimulated target cells (top) and traces from non-targets recorded across the mesoscale FOV (bottom). Adapted from (Abdeladim et al., 2023).

Finally, the extension of 2P all-optical approaches in freely moving animals is needed to study the causal link between activity patterns at the circuit level and behavior in more ethologically relevant behaviors. In 2023, the Emiliani group proposed the first two-photon endoscope (2P-FENDO), which utilizes a fiber bundle and a gradient index (GRIN) lens to achieve 2P imaging and 2P holographic photostimulation of multiple cells in freely moving animals (Figure 15).

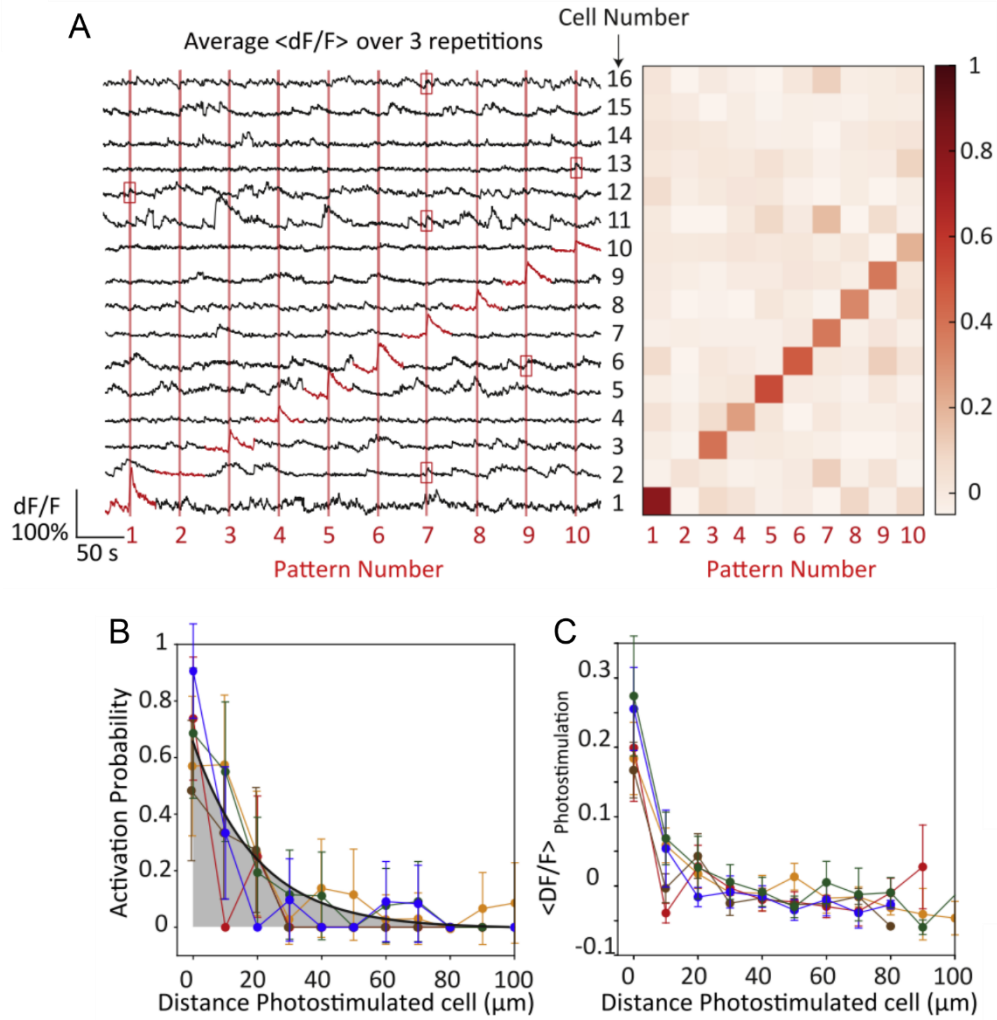


Figure 15. All-optical experiments in freely moving mice

A. Sequential photostimulation of 10 different neurons in freely moving animals using 2P-FENDO. Left: average $\Delta F/F_0$ traces (3 photostimulation repetitions) for every neuron. Photostimulation time windows are shown as red vertical lines. Red rectangles highlight instances of increased activity for non-targeted neurons concomitant with the photostimulation period. Right: heatmap representing the average $\Delta F/F_0$ upon photostimulation for target (1–10) and control (11–16) neurons for each photostimulation pattern. **B.** Activation probability of neurons as a function of distance from the target cells. Black solid line is an exponential decay fit, gray region is the area underneath the fit. **C.** Evoked $\Delta F/F_0$ for neurons that show a positive response to the photostimulation as a function of the distance from the photostimulated cells. Each color corresponds to a different FOV. Adapted from (Accanto et al., 2023).

2. Rationale

Understanding the relationship between neural activity and cognitive function is a challenging quest that requires multiscale experimental approaches to be properly addressed (Buzsáki, 2010; Kandel, 2013). To monitor neural activity, neuroscientific research has long focused on the one side on single-neuron and molecular characterization (Adrian, 1928, 1947; Hodgkin & Huxley, 1939; Hubel, 1957; Hubel & Wiesel, 1962; Sakmann & Neher, 1984), and on the other side on large scale non-invasive methods for the investigation of brain activity (e.g., EEG, fMRI; Buzsáki et al., 2012; Nunez & Srinivasan, 2006). While these approaches have drawn important insights, they do not allow for recording the activity of neuronal microcircuits, increasingly proposed as building blocks of brain cognitive functions (Buonomano & Maass, 2009; Buzsáki, 2010; Eichenbaum, 2018; Saxena & Cunningham, 2019; Yuste et al., 2024).

How does information encoded in specific activity patterns of given brain microcircuits relate to cognitive function? How is this information exchanged between neuronal circuits and how does it affect behavior? These questions are particularly difficult to address: neuronal circuits form complex architectures where anatomical, transcriptional, electrical, and chemical features of single cells densely connected in the three spatial dimensions shape spatial and temporal microcircuit activity dynamics (Bialek et al., 1991; Gouwens et al., 2019; Kandel, 2013; Liu et al., 2024; London et al., 2010; Rieke, 1999; Yao et al., 2023). Advanced optical imaging methods such as two-photon microscopy (Denk et al., 1990, 1996; Svoboda & Yasuda, 2006), coupled with genetically-encoded fluorescent sensors (Chen et al., 2013; Dana et al., 2016, 2019; Fink et al., 2024; Grienberger & Konnerth, 2012; Miyawaki et al., 1997a; Mohr et al., 2020; Nakai et al., 2001; Yokoyama et al., 2024; Zhang et al., 2023), enable us to record and perturb the activity of groups of neurons at cellular resolution (all-optical approaches),

contributing to disentangle the relative contribution of specific cellular subpopulations to specific cognitive functions (Carrillo-Reid et al., 2019; Dal Maschio et al., 2017; Marshel et al., 2019; Russell et al., 2024; Dalglish et al., 2020; Gill et al., 2020). In all-optical approaches, neurons must express two different types of proteins: calcium sensors, used as proxies of electrical activity (Denk et al., 1994; Grienberger & Konnerth, 2012), and opsins, light-sensitive molecules that, upon illumination, can cause neuronal excitation or inhibition (Boyden et al., 2005; Deisseroth, 2015; Yizhar et al., 2011; F. Zhang et al., 2011). Two-photon all-optical experiments typically involve a combination of blue-light-sensitive calcium indicators (e.g., GCaMPs; Chen et al., 2013; Dana et al., 2019; Zhang et al., 2023) and red-light-sensitive opsins (e.g., C1V1, ChRmine, and ChrimsonR; Yizhar et al., 2011; Marshel et al., 2019; Klapoetke et al., 2014). However, red-shifted opsins typically display a blue-shifted shoulder in their absorption spectra, partially overlapping with the absorption spectra of blue-shifted fluorescent indicators (Sridharan et al., 2022). This overlap may lead to unwanted neuronal depolarization during imaging periods (Akerboom et al., 2013; Forli et al., 2018; Packer et al., 2015; Rickgauer et al., 2014), resulting in biased results. To address this limitation of current all-optical approaches, my PhD work aimed to develop a crosstalk-free all-optical method combining high-efficiency two-photon manipulation using the blue-light-sensitive opsin stCoChR (Forli et al., 2021) and two-photon imaging using the high-performance, red-shifted indicator RCaMP3 (Yokoyama et al., 2024) in layer 2/3 excitatory neurons of primary somatosensory cortex (S1) of awake head-fixed mice. Using this experimental strategy, we achieved: i) recording of network activity with a larger dynamic range and sensitivity compared to previously used red-shifted indicators (Forli et al., 2021); ii) effective photo-activation of neurons with low average power per cell; iii) reduced crosstalk between imaging

and photostimulation; iv) effective co-expression of opsin and indicator using a bicistronic construct (in collaboration with the Sakamoto research group in Kyoto University); v) description of how the 2P photostimulation effect is influenced by neuronal network dynamics and the animals' arousal level.

The novel experimental approach developed in my PhD work will enable high-efficiency recording and manipulation of neuronal networks *in vivo* with effective spectral separation between the excitation spectra of the opsin and the calcium indicator. This novel all-optical method represents a powerful technological improvement to understand how neuronal network activity is related to sensory perception, motor actions, internal states, learning and memory, feeding, and social behaviors, consequently informing drug discovery and delivery studies for better treatments of brain disorders.

3. Materials and Methods

3.1 Animals

All experiments involving animals were approved by the IIT Animal Welfare Body, by the National Council on Animal Care of the Italian Ministry of Health (authorization 1084/2020-PR), and performed following guidelines established by the European Communities Council Directive. C57BL/6J and CaMKII α -Cre mouse strains were used. A maximum of 4 mice were housed in standard ventilated cages with access to food and water *ad libitum* in a 12-hour light-dark cycle. Experimental procedures were conducted on male animals older than 8 weeks. The number of animals used for each experimental dataset is reported in the text or in the figure legends.

3.2 Adeno-associated viruses (AAV)

The stCoChR construct (ssAAV5/2-hEF1 α -DIO-stCoChR_2A-NLS_EGFP-WPRE-hGHp(A); identifier: v933-1) deposited at the Viral Vector Facility (VVF) of the Neuroscience Center Zurich (ZNZ) (<https://www.vvf.uzh.ch/en.html>) by Ofer Yizhar (Weizmann Institute of Science, Rehovot, Israel) was purchased from the VVF-ZNZ. The RCaMP3 construct (AAV2/1-eSyn-RCaMP3-WPRE; Yokoyama et al., 2024) was kindly provided by Prof. Masayuki Sakamoto (Institute for Frontier Life and Medical Sciences, Kyoto University, 606-8507 Kyoto, Japan). The bicistronic construct expressing RCaMP3 and stCoChR (AAV2/1-CaMKII α -RCaMP3-P2A-stCoChR-HA-WPRE) was developed by Prof. Sakamoto in a collaborative effort. Viral constructs used in this work are reported in Table 1 (see below).

3.3 Viral injections and surgeries

Animals used in two-photon calcium imaging or all-optical experiments received the viral injection and the optical window implantation in two separate surgeries interleaved by a 7 – 10 days period, in which they were allowed to recover. Mice recovered for one more week after the second surgery, before the handling and habituation stages began (Gentet et al., 2010). Mice used in electrophysiological experiments under anesthesia underwent the surgery on the day of the experiment (see below).

3.3.1 Viral injections

Mice were anesthetized via inhalation of 4% isoflurane / 0.8% oxygen at a flow rate of 1 ± 0.2 L/min and then placed onto a stereotaxic apparatus (Stoelting Co, Wood Dale, IL). Dexamethasone (4 mg/kg, diluted 1:10 in saline; Dexadreson, MSD Animal Health, Milan, Italy) was administered intramuscularly. Eye ointment (Epigel, Trebifarma, Genova, Italy) was applied to prevent corneal desiccation. Isoflurane rate was kept at 0.8-1.5 L/min during surgery. Depth of anesthesia was monitored by checking respiration rate, whisker movement, and reactions to tail, foot, or ear pinching. Animals were kept at a constant temperature of 37°C with a heating pad (RS, Milan, Italy) throughout the surgical procedure. Lidocaine was injected under the scalp. The head was shaved and disinfected with betadine before exposing the skull with a small antero-posterior skin incision. The periosteum was removed with hydrogen peroxide and a cotton swab. Two to four injection sites were drilled on the skull over the right primary somatosensory cortex (S1). A variable quantity of viral solution (500 to 800 nL) was slowly injected (0.25 – 0.35 mm deep, 50 nl/min) in each injection site via a hydraulic injection apparatus driven by a syringe pump (UltraMicroPump, World Precision Instruments, Sarasota, Florida, USA). After each injection, the glass pipette was left in place for 3 to 5 minutes and

slowly retracted at the end of the injection procedure. Once the pipette was removed, the incision was sutured, and antibiotic cream (IruXol, Smith & Nephew, United Kingdom) was applied over the skin. Baytril (100 μ L, diluted 1:10 in saline; BAYTRIL, Bayer, Germany) was administered intraperitoneally. Animals were then housed in a heated cage system until they had fully recovered from anesthesia.

3.3.2 Chronic optical window preparation

The chronic optical window and the stainless steel headbar implantation surgery were performed 7 to 10 days after the viral injection. The surgical procedure mostly followed the protocol described above (see also Forli et al., 2018, 2021). The skin above the skull was completely removed, and the periosteum was removed with hydrogen peroxide and a cotton swab. After locating the injection sites, a craniotomy (3 mm in diameter) was executed. Two glass coverslips (3 mm and 4 mm in diameter; Crisel Instruments, Rome, Italy) previously glued together using optical glue (NOA 63; Norland Products, Jamesburg, USA) were then placed over the craniotomy area and fixed in place with epoxy glue (Loctite 454; Loctite, Düsseldorf, Germany). The skull was scratched with a scalpel before securing the headbar to it with super glue (Loctite Super Attack; Loctite, Düsseldorf, Germany). The implant was then secured with dental cement (Metabond adhesive cement; Parkell, Edgewood, NY, USA).

3.4 Optical setups

Two-photon imaging of RCaMP3-expressing mice was carried out on a different setup with respect to all-optical experiments. The description of the two setups employed is given below.

Optical experiments were always performed in awake, head-fixed mice, freely running on a wheel.

3.4.1 Optical setup for *in vivo* two-photon imaging of RCaMP3

Two-photon calcium imaging of head-fixed mice freely running on a wheel expressing RCaMP3 was performed using an Ultima Investigator scanhead (Bruker, Milan, Italy) equipped with resonant scanning galvanometers and a 16x/0.8 NA objective (Nikon, Milan, Italy). A Chameleon Ultra II tunable pulsed laser (80 MHz repetition rate, Coherent, Milan, Italy) was used as the excitation source. The beam intensity was then modulated with a Pockels cell (Conoptics, Danbury, Connecticut, USA). Fluorescence emission was collected by multi-alkali photomultiplier tubes equipped with a 525/70 nm filter for green-shifted emission and a 595/50 nm filter for red-shifted emission. Detector signals were digitized at 12 bits. All experiments were recorded in raster scanning mode at 30 Hz. The field of view dimension was always 512 pixels $\times$ 512 pixels (pixel dwell-time, 4 μ s; pixel size, 0.52 μ m) and acquired using a 3 $\times$ optical zooming factor (FOV dimension, $\sim$ 270 μ m $\times$ 270 μ m).

3.4.2 Optical setup for *in vivo* all-optical two-photon imaging and holographic stimulation

Two-photon all-optical experiments employed an upright epifluorescence microscope (BX61, Olympus, Milan, IT) with a customized body and scan-head (Bruker Corporation, Milan, IT). Two laser sources were employed: an ultrafast tunable pulsed laser (Chameleon Discovery, 80 MHz repetition rate, Coherent, Milan, IT) set at 1100 nm wavelength to image RCaMP3-expressing neurons, and a high-energy femtosecond laser (Carbide 45 W: Light conversion,

Vilnius, Lithuania) coupled to a non-collinear optical parametric amplifier (NOPA; Orpheus-F by Light conversion, Vilnius, Lithuania) to perform holographic spiral stimulation of neurons co-expressing RCaMP3 and stCoChR. The NOPA was used to shift the Carbide output wavelength (1035 nm) to 920 nm. Repetition rate of the high-energy laser was 1 MHz (Forli et al., 2021). Two Pockels cells (Conoptics Inc., Danbury, CT) were used to modulate the intensity of the two laser beams. The photostimulation optical path impinged onto a liquid crystal spatial light modulator (LC-SLM, X10468-07 SLM, Hamamatsu, Milan, IT), which was conjugated to the back aperture of the objective (Dal Maschio et al., 2010). Multiple mirrors (UM10AG, Thorlabs, Newton, NJ) were deployed to direct the high-energy beam onto the SLM display. A half-wave plate (RAC 5.2.10 achromatic $\lambda/2$ retarder - B. Halle Nachfl GMBH, Berlin, DE) granted the required light polarization, allowing phase-only modulation at the SLM (Bovetti & Fellin, 2015; Dal Maschio et al., 2010; Ronzitti et al., 2017; Y. Yang et al., 2023). The beam was expanded with a telescope (IR doublets 30 mm and 200 mm, Thorlabs, Newton, NJ) to match the laser beam diameter to the dimension of the SLM matrix. A second telescope (IR doublets 400 mm and 200 mm, Thorlabs, Newton, NJ) was placed downstream the SLM to decrease the laser beam diameter to fit the size of the galvanometric mirrors (mirror diameter: 6 mm) inside the scan-head. After the scan lens and tube lens, a 660 nm long-pass dichroic mirror was placed to allow transmission of infrared light (IR) and to reflect the emitted fluorescence from the sample. A 575 nm long-pass dichroic mirror was placed after the D1 dichroic mirror to direct the emitted fluorescence in two different multi-alkali photomultiplier tubes (PMTs, Hamamatsu, Milan, IT). Both PMTs were equipped with appropriate emission filters (525/70 nm for the green channel, 607/45 nm for the red channel). A 980 nm long-pass dichroic mirror (zt980rdc, Chroma Technology Corporation, Bellows

Falls, VT) was placed upstream the scan lens to combine the imaging and stimulation laser beams. The calibration procedure to match the imaging and stimulation field of views and the generation of phase masks for holographic stimulation were done as previously described (Forli et al., 2018; Forli et al., 2021). The weighted variant of the Gerchberg-Saxton algorithm (WGS, Di Leonardo et al., 2007) was employed when generating phase masks to compensate for the reduction in power efficiency at different diffraction angles. The laser average power was measured below the objective with a commercially available power meter (Thorlabs, Newton, NJ). All experiments employed a 16x/0.8 NA objective (Nikon, Milan, Italy).

3.5 All-optical experiments

3.5.1 All-optical experiments and behavioral monitoring

Simultaneous two-photon imaging and photostimulation experiments and behavioral monitoring (see section 3.7 for details on locomotion and pupil dilation recordings) were conducted on awake head-fixed CaMKII-cre mice freely running on a wheel co-expressing the calcium sensor RCaMP3 and the opsin stCoChR in L2/3 excitatory neurons of primary somatosensory cortex (S1), 4 to 6 weeks after virus injection. Mice were tested over consecutive days, and, every day, a different number of neurons was photostimulated (number of stimulated neurons: 1, 3, 5, or 10). An experimental session started with the assessment of indicator and opsin expression levels by acquiring high-resolution images at 1100 nm and 920 nm, respectively. In this way, a combined reference image of a selected field of view could be generated to identify co-expressing neurons and calculate the phase mask for holographic stimulation. Short temporal series (t-series) were then acquired while 5 to 15 manually selected target cells were simultaneously photostimulated at 920 nm to evaluate their responsivity over

~10 photostimulation trials. Neurons showing photostimulation responses with high SNR were selected. For every experiment, three t-series (imaging laser power: 40 – 70 mW; 2× optical zoom, 256 × 256 pixels; pixel dwell-time, 4 μs; pixel size, 1.51 μm) each lasting ~12 minutes were acquired at ~3 Hz. Over one t-series, 60 photostimulation trials were delivered, one every ~10 seconds, for a total of 180 trials obtained per experiment. Every recording started and ended with a one-minute window without photostimulation trials. Successive t-series were interleaved by a 10 minute time interval without imaging or stimulation. Mice were free to run on a wheel throughout the experimental session.

3.5.2 All-optical experiments with bicistronic construct

All-optical experiments were conducted on awake head-fixed C57BL/6J mice freely running on a wheel previously injected in L2/3 of S1 with the bicistronic virus, which carries the coding sequences for RCaMP3 and stCoChR under the CamKIIα promoter. Experiments started from two months post-injection, consisting of the repeated photostimulation (n = 60 events) of a neuronal ensemble (number of targets: 8 – 10 neurons per session; stimulation parameters as described in 3.5.3) selected within a ~270 x 270 μm FOV (imaging laser power: 50 – 70 mW; 2× optical zoom, 180 × 180 pixels; pixel dwell-time, 4 μs; pixel size, 1.51 μm).

3.5.3 Photostimulation protocol

Single cells were photostimulated via holographic spiral stimulation. Every stimulation trial consisted of 5 spirals lasting 20 milliseconds each, delivered consecutively from the center of the cell outward, for a total stimulation duration of 100 milliseconds. Spiral diameter was 10

μm , average power delivered per cell was 5 mW. Photostimulation began at the start of the frame, every ~ 30 frames (~ 10 seconds).

3.6 Behavioral monitoring: locomotion and pupil size

Mice could freely run on a wheel along the rostro-caudal axis throughout the experimental sessions. Wheel rotations were detected with an optical rotary encoder (RS Components S.r.l., Sesto San Giovanni, Milan, Italy) with a resolution of 2000 counts/revolution and 100 Hz sampling rate. Images ($112 \text{ pixels} \times 100 \text{ pixels}$) of the eye contralateral to the cranial window were recorded with a high-speed camera and dedicated software (model: acA800-510 μm , Basler AG, Ahrensburg, Germany; PylonViewer Software) in frame burst mode, i.e., each microscope frame triggered the acquisition of 9 frames at 30 Hz. The eye was illuminated with an infrared light-emitting custom diode, as the IR light transmitted from the pupil during two-photon imaging at 1100 nm wavelength was insufficient to clearly identify the pupil. Pupil recordings were analyzed with SLEAP, an open-source deep learning-based model for automatic movement tracking (Pereira et al., 2022).

3.7 Data analysis

Two-photon optical imaging and photostimulation data were analyzed using CITE-On (Sità et al., 2022) and Python custom scripts (version 3.7). Blinding was not used in any of the experiments presented in this work. Recordings presenting technical issues were excluded from the analysis.

3.7.1 Optical recordings preprocessing

Every t-series consisted of a sequence of images in .tiff format. These images were first combined into a multi-tiff file with a custom script. Each t-series was motion corrected employing up-sampled phase cross-correlation (Guizar-Sicairos et al., 2008; Van der Walt et al., 2014) as in (Curreli et al., 2022), using its median projection as reference template. Then, to minimize lateral motion artifacts across recordings belonging to the same experimental sessions, single corrected t-series were concatenated into a single movie, and motion correction was run a second time using the median of single median projections as a reference. Concatenated t-series in the same experimental session were treated independently in subsequent preprocessing steps and analyses. Generation of bounding boxes, cell detection, and trace extraction were performed using CITE-On (Sità et al., 2022), with modifications to the pipeline done with the contribution of Leonardo Nacci to accommodate the specific image acquisition features of the all-optical experiments described in this thesis work. Briefly, CITE-On increases original signal-to-noise ratio of single frames by averaging consecutive ones, and performs upscaling of the resulting projection to achieve optimal size of the t-series to be later processed by the convolutional neural network (CNN) employed to detect cell bodies and generate bounding boxes around each cell. After performing cell detection with CITE-On, a .png output file from the custom software controlling the SLM (Labview, National Instruments Corp, Austin, TX) displaying the target points defined during the experiments was overlaid onto the CITE-On detections. This new step enabled reliable identification of target neurons by calculating the Euclidean distance between each target point and the centroid of each bounding box, and classifying a cell as a target if it was closest to a given target point. Post-hoc visual inspection was carried out to remove clear false detections and/or to add any obvious missed cells. Traces were then extracted from the final sets of detected cells. To decontaminate

the fluorescence traces from the neuropil signal, CITE-On preprocessing was modified to subtract from each ROI a local background signal computed as the intensity values between the 10th and 25th percentile of the ROI intensity values in all recordings. The native version of CITE-On performs neuropil correction by subtracting a global background value computed outside

The change in fluorescence relative to the baseline ($\Delta F/F_0$) was computed as a function of time. The baseline fluorescence (F_0) was calculated either in ~ 1 second preceding each photostimulation trial for photostimulation responses analyses (experiments in figures 18, 19, 23, 24, 27, 28, 32) or as the median value of the fluorescence values below the 30th percentile of the whole trace (experiments in figures 20, 31). The latter method was chosen over the rolling window method to preserve the magnitude of the photostimulation responses and the long-time courses of fluorescence traces. The rolling window method was employed only for 2P calcium imaging recordings in mice expressing RCaMP3 only (Figure 17), with F_0 computed as the 20th percentile of the average fluorescence intensities in a 10 seconds rolling window centered at t .

3.7.2 Calcium events analysis

For the analysis presented in Figure 17, statistically significant calcium events were extracted from $\Delta F/F_0$ traces as in Curreli et al. (2022). To extract calcium events from neuronal traces, the standard deviation of the whole trace (σ_1) was first computed. Values exceeding $\pm \sigma_1$ were removed, and a second standard deviation (σ_2) was calculated on the thresholded trace. This approach allows to reduce bias from large transients and yields a more accurate estimate of the baseline (σ_2). Fluorescence events were then detected on the original trace as transients that (i)

crossed $\pm 3\sigma_2$ and (ii) returned within $\pm 2\sigma_2$ within 0.5 seconds. The bona-fide minimum duration of a calcium event was set at 0.5 seconds based on reported off-kinetics of RCaMP3 (Yokoyama et al., 2024).

3.7.3 Behavioral parameters: treadmill and pupillometry

After conversion of rotations to speed, we classified an time instant as part of a running period, if: i) instantaneous speed was >1 cm/s, ii) filtered speed with centered rolling average (window size: $\text{fps} / 0.25$) was >1 cm/s, iii) average speed over a 2-second time window was > 0.1 cm/s. Temporal gaps between running periods shorter than 0.5 s were considered periods of locomotion. Locomotion epochs were then binned into three different speed ranges (1 – 10 cm/s, 10 – 20 cm/s, ≥ 20 cm/s), classified as low, medium, or high velocity similarly to (Saleem et al., 2018).

3.7.4 Quantification of target neurons' responsiveness

A target neuron was defined as responsive to photostimulation (Figure 18, 31, 32) if the mean peak post-stimulus activity in a 500-millisecond time window across delivered photostimulation events ($n = 60$) exceeded a threshold set as the 95th percentile of the distribution of all $\Delta F/F_0$ values in the 1-second window preceding each photostimulation event.

3.7.5 Effect of the stimulation on network neurons

To determine the impact of target neurons photostimulation on network cells, an influence metric was computed for each non-target neuron (Figures 25, 26, 29, 30, 32; Khoury et al., 2025). For each photostimulation trial, the mean fluorescence in the Pre-stimulation and

Stimulation windows (~640 ms) was computed. The difference between the two (Δ) was normalized by the standard deviation of Δ across photostimulation trials. The mean of these differences (influence score) reflects the strength and consistency of the neuron's response relative to its variability. The statistical significance of the influence score was then evaluated using a permutation test under the null hypothesis that changes were random in sign. A null distribution of mean differences was produced by randomly flipping the signs of Δ for each neuron across trials ($n = 10,000$ permutations). The percentage of permuted means with absolute magnitude larger than or equal to the observed mean was used to determine the p-value. Non-target neurons were then classified as excited (influence score > 0 , $p < 0.05$), inhibited (influence score < 0 , $p < 0.05$), or neutral (all remaining cells).

3.8 Statistical tests

All values were expressed as mean $\pm$ SEM, unless otherwise noted. The significance threshold for test statistics was set at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Data was tested for normality with a Kolmogorov-Smirnov ($n \geq 10$) or Shapiro-Wilk ($n < 10$) test. All data were not normally distributed. As such, non-parametric tests were applied. When comparing two populations of data, the Wilcoxon signed-rank test (paired) or the Mann-Whitney test (unpaired) with post hoc Dunn's test was used. For multiple comparisons (> 2 populations), the non-parametric Friedman test (paired) or the Kruskal-Wallis test (unpaired) with post hoc Dunn's test was used. Multiple comparison corrections were performed using the Bonferroni method. All tests were two-sided. Python custom scripts (version 3.7) were used to perform all statistical analyses.

4. Results

4.1 A high-performance red-shifted indicator for all optical experiments with minimal crosstalk

The combination of a red-light-sensitive indicator (e.g., jRCaMP1a) with a blue-light-sensitive opsin (e.g., ChR2) significantly decreases the optical crosstalk between 2P calcium imaging and 2P optogenetic activation in all optical 2P imaging and perturbation experiments (Forli et al., 2018). The introduction of the large-conductance opsin stCoChR (Forli et al., 2021; Klapoetke et al., 2014) and the use of low-repetition rate laser sources enables efficient optogenetic excitation with minimal light power (few mW average power per cell; Forli et al., 2021). However, the choice of a blue-shifted opsin and a red-shifted indicator suffers from the limited SNR, dynamic range, and slow kinetics typical of red-shifted indicators compared to the blue-shifted ones (Chen et al., 2013; Dana et al., 2016; Forli et al., 2018, 2021; Zhang et al., 2023). Yokoyama and colleagues (2024) recently engineered RCaMP3, a new red-shifted calcium indicator with rise and decay times comparable to the widely used red indicator jRGECO1a, a larger dynamic range ($\Delta F/F_0$), and enhanced sensitivity in detecting spike-dependent calcium activity. RCaMP3 was reported to be excitable through a two-photon excitation process, but its two-photon absorption spectrum has not been reported (Yokoyama et al., 2024; see figure 16A for 1P excitation and emission spectra). We therefore first set up to measure its action spectrum using two-photon microscopy on fixed brain slices expressing RCaMP3 in cortical neurons (Figure 16B). RCaMP3 was expressed in the brain tissue using intraparenchymal viral injections in young mice (see Methods for details). We found that the action spectrum peaked at ~ 1060 nm wavelength. At 920 nm, the normalized action spectrum

value was ~58% of the peak excitation, and at 1100 nm, the wavelength previously used for all optical experiments using red-shifted indicators, was ~59% (Figure 16B).

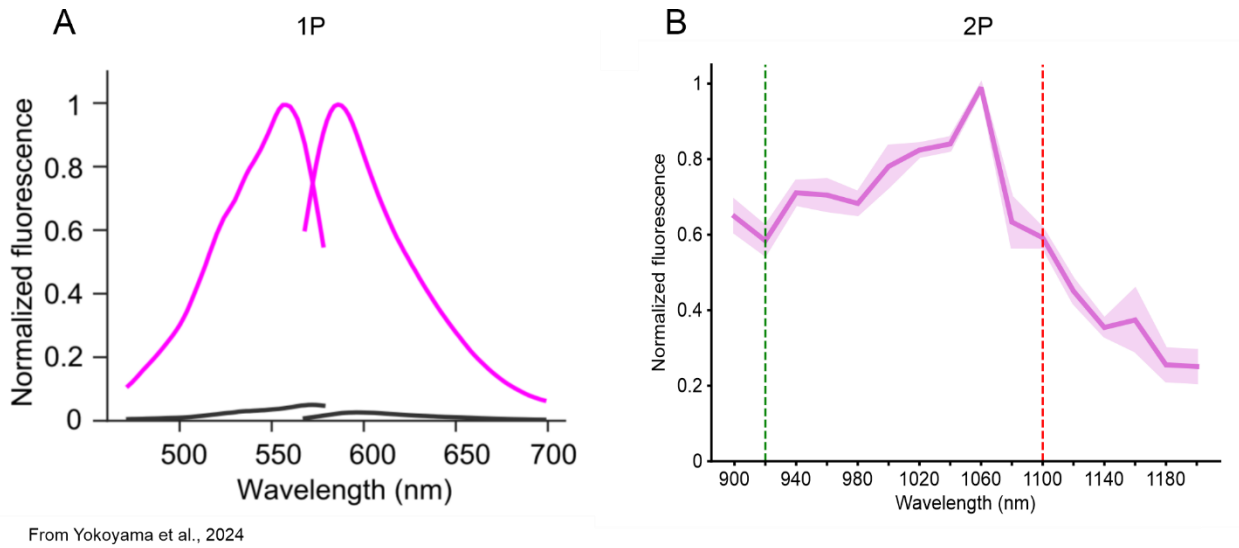


Figure 16. One- and two-photon spectral properties of RCaMP3

A. One-photon excitation and emission spectra of RCaMP3 in Ca^{2+} -free and Ca^{2+} -saturated states measured in HEK cell lysate (adapted from Yokoyama et al., 2024). **B.** Two-photon excitation spectrum of RCaMP3 measured on fixed brain slices of mice expressing RCaMP3 in neurons of the primary somatosensory cortex ($n = 3$ slices from $m = 3$ animals).

Given its superior performance compared to other available red-shifted calcium indicators, we aimed to co-express RCaMP3 with stCoChR to achieve unprecedented readout capabilities in an all-optical 2P experimental configuration, which minimizes crosstalk between imaging and photostimulation. Yokoyama and colleagues performed two-photon calcium imaging experiments in primary visual cortex (V1) at the excitation wavelength of 1040 nm (close to the peak of the two-photon action spectrum, Figure 16B). Given that imaging the red-shifted indicator at 1100 nm reduces spectral crosstalk (Forli et al., 2018, 2021), we first investigated whether we could image RCaMP3-expressing neurons with sufficient SNR at longer wavelengths (i.e., > 1040 nm). We injected RCaMP3 into layer 2/3 of the primary

somatosensory cortex and performed two-photon calcium imaging on awake, head-fixed mice at different wavelengths (1040 nm, 1060 nm, and 1080 nm; Figure 17). We confirmed high levels of expression of RCaMP3, and we observed high SNR RCaMP3 signals over time at all tested wavelengths (1040 nm, 1060 nm, and 1080 nm; Figure 17B, C). We did not observe evidence of photobleaching at longer wavelengths (duration of recordings: 2 minutes, Figure 17D), in agreement with what was reported by Yokoyama et al. at 1040 nm. We therefore opted to set our imaging wavelength to 1080-1100 nm, a range previously shown not to activate stCoChR. Please note that normalized mean fluorescence values of RCaMP3 at 1080 nm and 1100 nm are similar (~59% at 1100nm, ~63% at 1080 nm; Figure 16, 17; Forli et al., 2021). Quantification of changes in average fluorescence values between the start and the end of the recordings revealed a small but significant increase in fluorescence at the end of recordings performed at 1040 and 1080 nm (1040 nm, change at the end of the recording: +4.47 a.u. with respect to mean fluorescence values at the start of the recording; 1080 nm, change at the end of the recording: +14.77 a.u.; Figure 17D). This result tends to exclude the possibility that RCaMP3 undergoes photobleaching under our experimental conditions

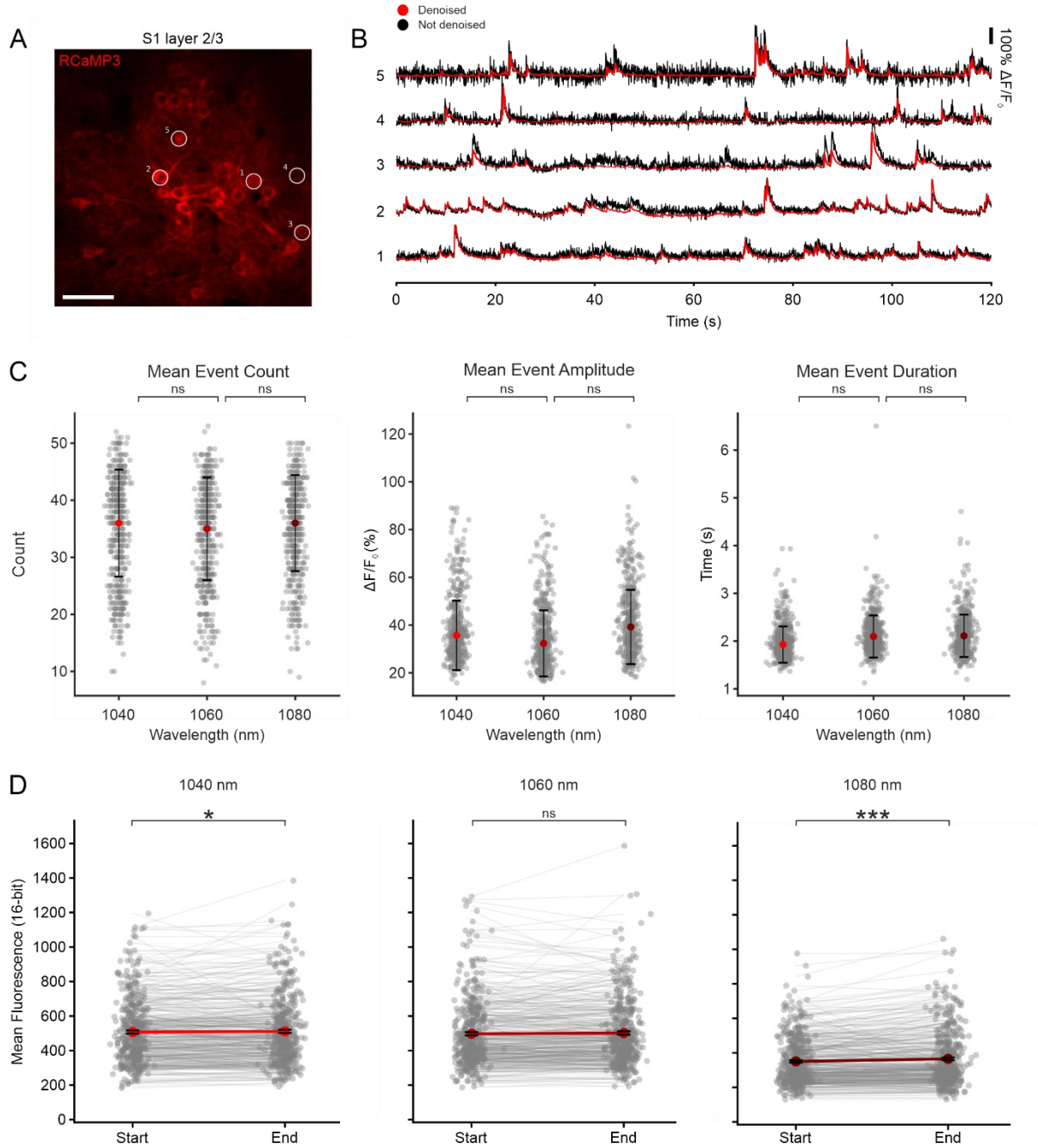


Figure 17. Functional expression of RCaMP3 in layer 2/3 of the mouse primary somatosensory cortex

A. Representative field of view of layer 2/3 of S1 recorded with 2P fluorescence microscopy in an awake head-fixed mouse ($\lambda_{\text{exc}} = 1080 \text{ nm}$; Power on sample = 50 mW). Scalebar: 50 μm . **B.** $\Delta F/F_0$ values of functional fluorescence traces (black) and denoised traces (red) from the five ROIs indicated in A (imaging acquisition rate = 30 Hz). Analyses were performed on non-denoised data. **C.** Average event count (left), amplitude (middle), and duration (right) for single neurons (grey dots). The same cells ($n = 140$ cells from $m = 2$ animals) were recorded at three different wavelengths. Friedman test with Dunn's post hoc test (ns, $p > 0.05$). Red dots indicate

median values, error bars indicate standard deviation. **D.** Comparison of average raw fluorescence values for single cells at Start (10 sec) and End (10 sec) of the recording under 1040 nm (left), 1060 nm (middle), and 1080 nm (right) imaging wavelengths. Wilcoxon signed-rank test with Bonferroni correction for multiple comparisons (1040 nm, $p = 0.014$; 1080 nm, $p = 4.9\text{e-}7$)

4.2 Functional co-expression of RCaMP3 and stCoChR using two independent viral vectors

After having confirmed that RCaMP3 is an efficient calcium indicator for 2P imaging at the longer wavelength (e.g., 1100 nm) required to minimize crosstalk in all optical 2P imaging and perturbation experiments, we aimed to co-express RCaMP3 with stCoChR in layer 2/3 principal cells of S1 using a Cre-lox approach (Figure 18). We used the transgenic mouse line CamKII-Cre, which expresses the enzyme Cre recombinase in a large number of brain cells, including principal neurons of S1, and we injected 10-week-old CamKII-Cre mice with an AAV virus carrying the RCaMP3 construct and a second virus carrying the stCoChR construct. Two-photon microscopy experiments were conducted in awake mice freely running on a wheel (Antonini et al., 2020; Sità et al., 2022; Vecchia et al., 2020; Figure 18A). First, the field of view was visually assessed for co-expression. We observed co-expression of the two proteins 4 weeks post-injection, with neurons expressing the constructs showing no evident signs of abnormal morphology or degeneration (Figure 18B). It is important to note that the stCoChR construct employed here was designed with a nuclear localization sequence (NLS-eGFP), which permitted nuclear tagging with eGFP protein of stCoChR-expressing neurons (see section also 3.2). This experimental strategy facilitated the identification of neurons co-expressing the opsin (green staining in the cell nucleus) and the calcium indicator (red staining in the cell cytoplasm). We next used the high repetition rate (80 MHz) laser source to image

RCaMP3 ($\lambda_{\text{exc}} = 1100 \text{ nm}$) and confirmed high SNR spontaneous fluorescence transients in L2/3 neurons of S1. We then moved on to perform all optical experiments and used a second laser source with low-repetition rate (1 MHz, $\lambda_{\text{stim}} = 920 \text{ nm}$) for two-photon holographic stimulation of stCoChR in randomly selected ensembles of neurons (Figure 18). A region of interest (ROI, $164 \times 164 \mu\text{m}^2$) was identified on the FOV for imaging and raster scanned at 17 Hz, similar to experiments carried out in Forli et al. (2021) (Figure 18B). Randomly selected co-expressing neurons (~ 10 cells per ROI) were selected and repeatedly photostimulated every 5 seconds (Figure 18B, D). Holographic spiral scanning stimulation consisted of $5 \times 20 \text{ ms}$ spirals delivered at 20 Hz (total stimulation duration: 500 ms) with an average power per cell of 7 mW.

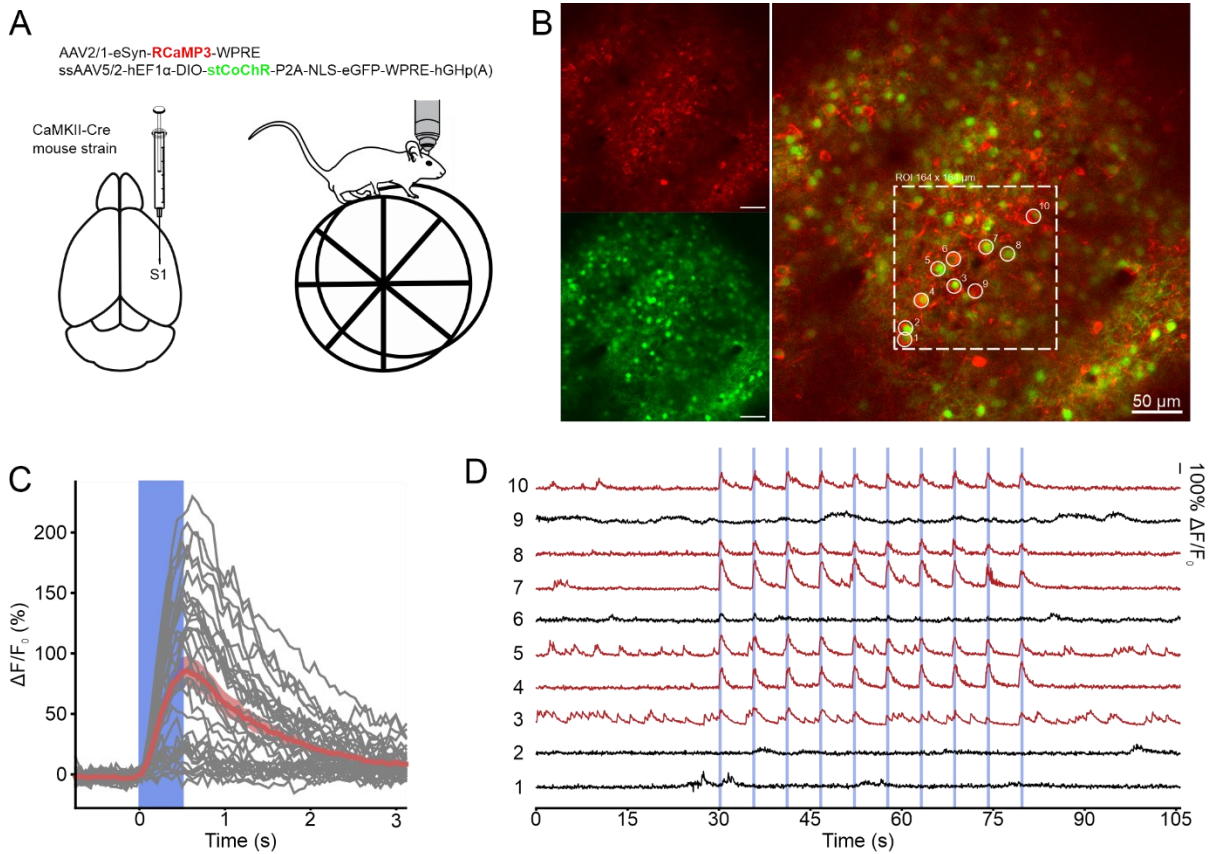


Figure 18. Co-expression of RCaMP3 and stCoChR in L2/3 cortical neurons of the mouse S1

A. Viral injection of two independent AAV viruses in S1 of CaMKII-Cre mice to achieve co-expression of RCaMP3 and stCoChR in principal neurons (left). Awake, head-fixed mice running on a wheel were tested starting from 4 weeks post-injection (right). **B.** Representative field of view showing layer 2/3 excitatory neurons co-expressing RCaMP3 and stCoChR in S1 (2 months post-injection). A ROI (164 x 164 μm^2 ; dashed white line) was selected for the all-optical experiment. Ten neurons (white circles) were targeted for photostimulation. Scale bar: 50 μm . **C.** Photostimulation-triggered average traces of neurons in all experiments (27 responsive neurons out of 37 photostimulated target cells). Average responses from single cells (grey) and mean activity across all cells $\pm$ SEM (red) are shown. The blue bar indicates the photostimulation time window. **D.** $\Delta F/F_0$ traces from the 10 target neurons tested in the experiment shown in B. 6 (red traces) out of the 10 photostimulated neurons showed large amplitude and reliable responses to holographic stimulation.

We observed reliable and high SNR changes in RCaMP3 fluorescence in a sizeable fraction of stimulated neurons (6 out of 10 in Figure 18B; across all experiments, we report 27 responsive neurons out of 37 photostimulated target cells), confirming functional expression and efficient stimulation of stCoChR by holographic illumination. Light-evoked events overlasted the duration of the light stimulus, with an average peak value of $111.9 \pm 0.1\%$ $\Delta F/F_0$ among 27 responsive neurons.

Photostimulation of co-expressing cells with light tuned at 920 nm may potentially excite fluorescence emission from RCaMP3 (Figure 16B) and/or from the eGFP fluorescent tag of stCoChR, which may partially affect the signal recorded in the red fluorescence channel (“bleed through”). Both effects could alter the amplitude and kinetics of the RCaMP3 response. To assess the presence of fluorescence artifacts induced by photostimulation, we repeated the photostimulation experiments selecting RCaMP3-only expressing cells in mice co-expressing RCaMP3 and stCoChR ($n = 3$ animals). We simultaneously photostimulated either 10 neurons recorded at 3.1 Hz (FOV dimension: $\sim 390 \times 390 \mu\text{m}$) or 5 neurons recorded at 9 Hz (FOV dimension: $\sim 390 \times 130 \mu\text{m}$). The photostimulation protocol had the following characteristics: 5 x 20 ms spirals, stimulation duration 100 ms, average power per cell 5 mW (see also section

4.3). In the first case (10 targeted cells per FOV recorded at 3.1 Hz; frame period, ~320 ms), the photostimulation artifact would affect about 1/3 of the image lines per frame. In the second case (5 targeted cells per FOV recorded at 9 Hz; frame period, ~110 ms), it would affect almost all image lines in each stimulation frame. We found no significant difference in fluorescence levels between Pre-, Stimulation, and Post-stimulation windows (each window consisting of 1 frame; ~320 ms at 3.1 Hz, ~110 ms at 9 Hz) under both conditions in raw and background-subtracted traces (Figure 19). There was, however, a tendency to increase in median fluorescence ($11.3 \pm 2.6\%$ for raw values, $8.5 \pm 3.0\%$ for background-subtracted values; $n = 30$ cells) between the Pre-stimulation and Stimulation windows in the condition entailing 10 targets recorded at 3.1 Hz (Figure 19B, D). This observation may lead to the conclusion that, although minimal, some photostimulation artifact is present. However, we cannot exclude the alternative hypothesis that cells that we considered RCaMP3-expressing only may have minimal stCoChR expression, which is not visible through GFP detection with two-photon microscopy, but may be responsible for a slight depolarization of cells upon photostimulation. To avoid potential contamination from photostimulation artifacts, in all subsequent analyses of time-series acquired at 3.1 Hz, we corrected the fluorescence signal by subtracting, for photostimulated cells, 20% of the fluorescence intensity measured in the frame containing the photostimulation.

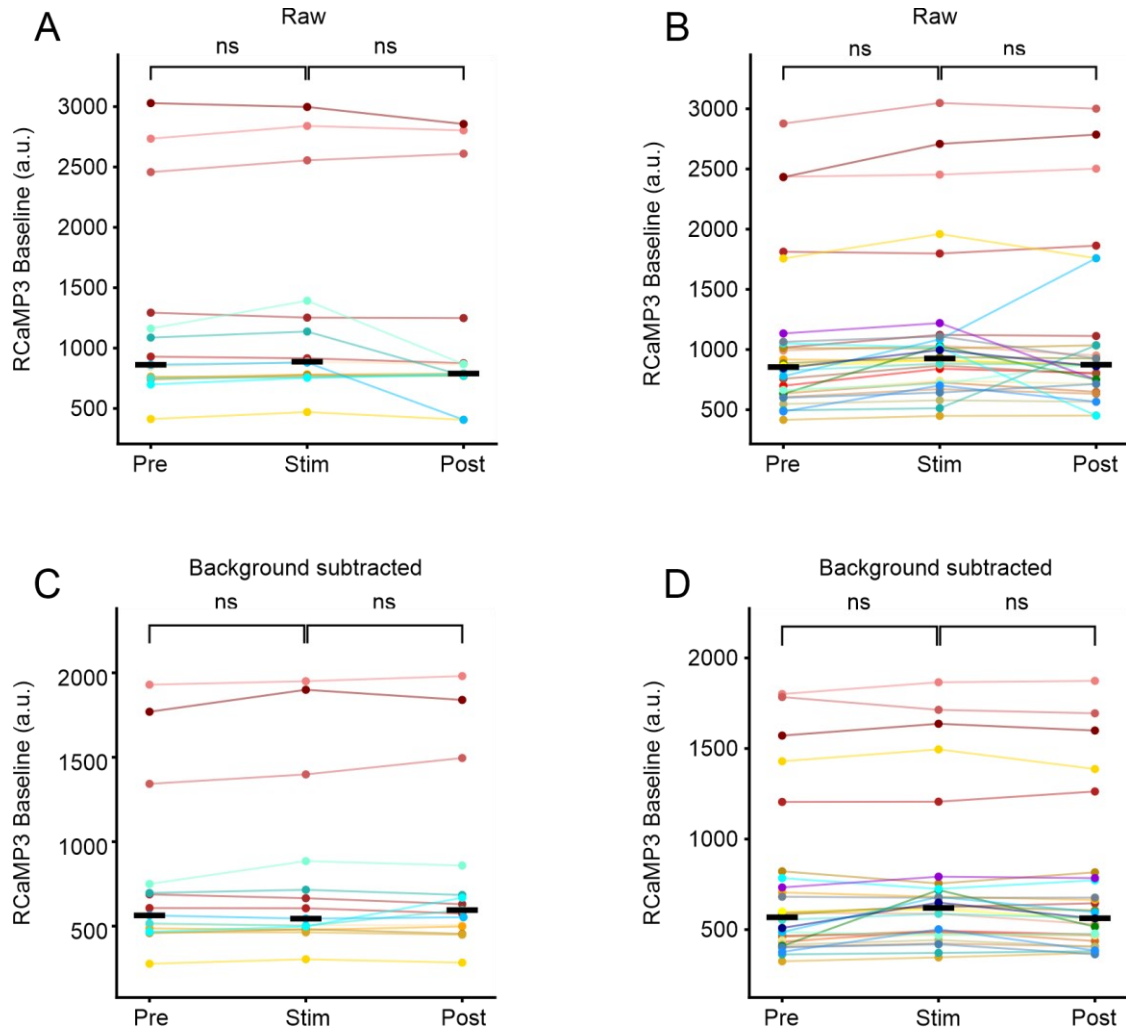


Figure 19. Evaluation of photostimulation artifacts on RCaMP3-only expressing neurons

A, C. Fluorescence intensity values in the Pre-, Stimulation, and Post-stimulation windows (1 frame) in raw fluorescence (**A**) and background-subtracted traces (**C**) recorded at 9 Hz (15 target neurons from 3 mice). Median values (black horizontal bars) are 862.0, 887.4, 789.3 for Pre, Stimulation, and Post windows respectively, in (**A**); 563.4, 544.3, 594.8 in (**C**). Friedman test (**A**, $p = 0.15$; **C**, $p = 0.93$), Dunn's test with Bonferroni correction for multiple comparisons ($p > 0.05$). **B, D.** Same as in **A, C**, but for recordings acquired at 3.1 Hz (30 target neurons from 3 mice). Median values (black horizontal bars) are 854.4, 926.1, 873.9 for Pre, Stimulation, and Post windows, respectively, in (**B**); 568.1, 619.8, 562.8 in (**D**). Friedman test (**B**, $p = 1.4e-05$; **D**, $p = 4.4e-3$), Dunn's test with Bonferroni correction for multiple comparisons (**B**, Pre vs Stim $p = 0.64$, Stim vs Post $p = 1.0$; **D**, Pre vs Stim $p = 0.82$, Stim vs Post $p = 1.0$).

4.3 Influence of network activity and arousal level on two-photon photostimulation-evoked responses

We next designed an experiment to test whether neuronal responses to holographic photostimulation would be affected by ongoing network activity (network state) and/or by the animal arousal level (animal state). We reasoned that this type of experiments would help understanding: i) whether and how evoked responses from target neurons would change depending on the animal arousal levels, and whether this phenomenon would show an interaction with the number of targets simultaneously stimulated; and ii) how network activity measured in non-target neurons would change as a function of the number of stimulated target cells, and how these changes in the activity of non-target neurons would be influenced by underlying arousal levels. To explore these possibilities, we set up to record locomotion in head-fixed mice running on a treadmill and to monitor pupil dilation (used as a proxy for arousal levels, (Joshi et al., 2016; Joshi & Gold, 2020; McCormick et al., 2020; Reimer et al., 2016), while simultaneously performing two-photon imaging and two-photon holographic photostimulation of either 1, 3, 5, or 10 cells simultaneously (Figure 20, 21).

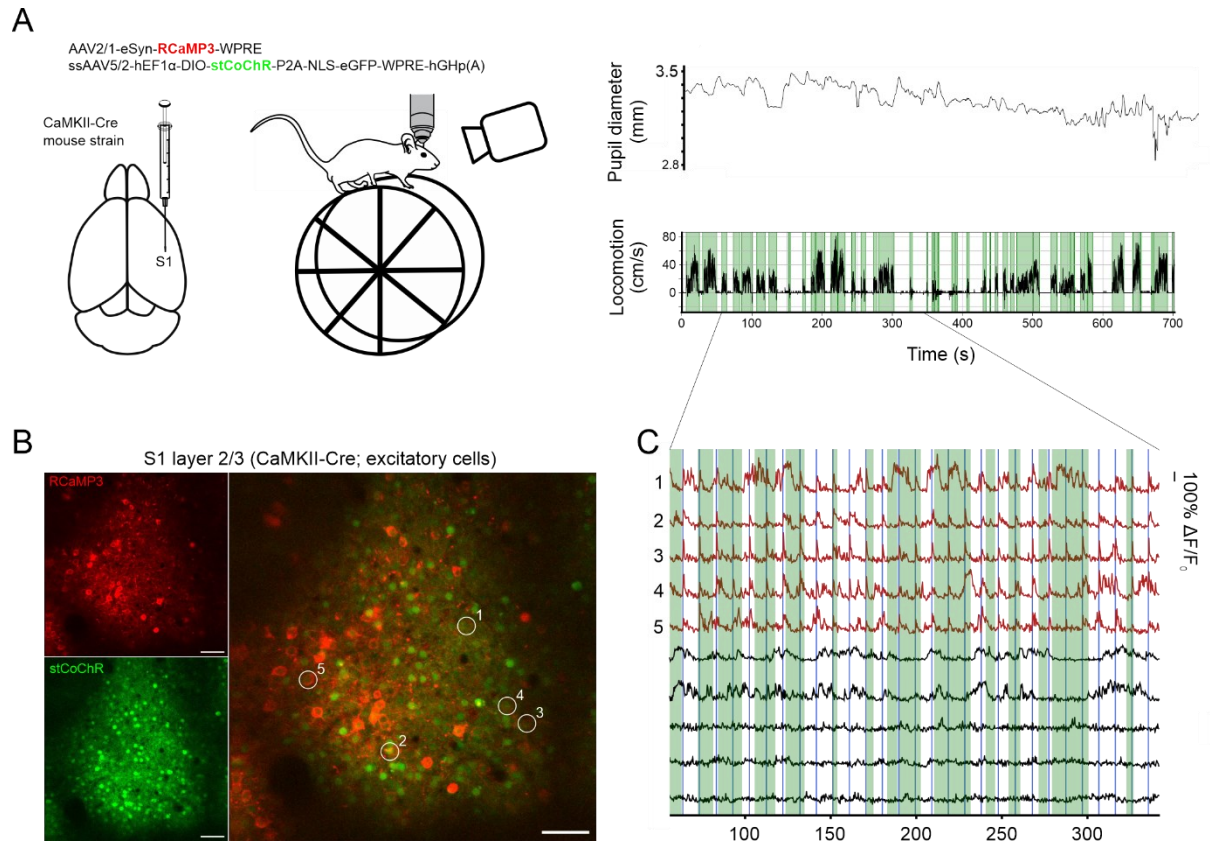


Figure 20. All-optical imaging and manipulation experiments as a function of locomotion and arousal

A. Experiment schematic. Head-fixed mice could freely run on a treadmill, and pupil dilation was recorded with a camera. Animals co-expressed RCaMP3 and stCoChR in excitatory neurons of L2/3 in S1. Green shaded areas indicate locomotion periods. **B.** Representative field-of-view displaying RCaMP3 expression (red) and stCoChR (monitored through the GFP nuclear tag, green). Five target neurons were repeatedly photostimulated every 10 seconds (white circles, numbers 1-5). **C.** Representative traces from the five target neurons (red) and five randomly selected non-target neurons (black). Blue bars on top indicate photostimulation time windows. Green shaded areas indicate locomotion periods.

For this set of experiments, we delivered 5 x 20 ms spirals (100 ms stimulation duration), with an average power per cell of 5 mW. For each condition (i.e., the different number of simultaneously targeted cells), we photostimulated neurons for 180 trials in an open-loop configuration.

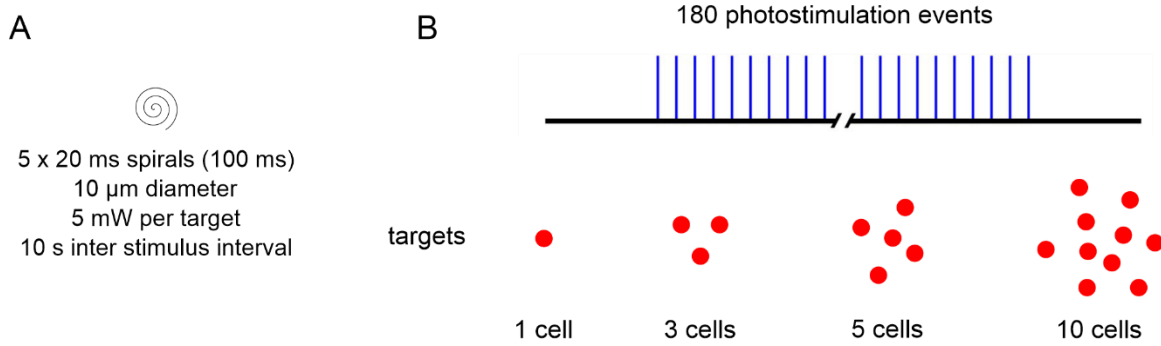


Figure 21. Illustration of photostimulation protocol

A. Spiral photostimulation trials (5 x 20 ms) were delivered every ten seconds. **B.** Each condition (i.e., number of targets) was tested on consecutive days in the same mice. For each condition, 180 stimulation trials were delivered, equally distributed over 3 different recordings, each lasting ~12 minutes and interleaved by 10-minute no stimulation windows. A short imaging period (1 minute) without photostimulation was also included at the beginning and at the end of every recording.

Before each experimental session, we identified and mapped photostimulation-responsive neurons within the selected field-of-view. The number of stimulated cells per field-of-view is displayed in Table 1.

	Condition (number of target cells)			
Animal fov	1 target	3 targets	5 targets	10 targets
1 – fov1				
1 – fov2				
2 – fov1				
3 – fov1				
4 – fov1				
5 – fov1				

Table 1. Summary of tested experimental conditions

All acquired field of views except one (1 - fov1) are missing at least one experimental session. In particular, the 10 targets condition has been tested only on 2 out of 6 field of views. Due to slight z refocusing in single field of views over different experimental session, FOVS were treated as independent in the analyses.

We first examined the effect of running activity on photostimulation responses. Throughout the experimental sessions, mice spent most of the time still (mean = 68.9%, SEM = 4.9%; n =

5 animals from $m = 17$ experimental sessions; Figure 22A) and only a minor portion locomoting (mean = 22.7%, SEM = 4.3%; $n = 5$ animals from $m = 17$ experimental sessions; Figure 22A). We subdivided the time spent locomoting into three different locomotion levels based on average speed (cm/s; Figure 22B), similarly to (Saleem et al., 2018), defined as “low” ($1 \text{ cm/s} < \text{speed} \leq 10 \text{ cm/s}$), “medium” ($10 \text{ cm/s} < \text{average} \leq 20 \text{ cm/s}$), and “high” ($\text{speed} > 20 \text{ cm/s}$). Mice spent a comparable amount of time running at these 3 speed ranges (low speed: mean = 7.4%, SEM = 1.1%; medium speed: mean = 8.3%, SEM = 1.7%; high speed: mean = 7.0%, SEM = 2.5%; $n = 5$ animals from $m = 17$ experimental sessions for all speed ranges; Figure 22B,C). The cumulative speed distributions calculated across mice for each condition (Figure 22C) show that locomotion speed was not evenly distributed across stimulation conditions. When stimulating 1 target cell, the full cumulative probability was shifted leftward, indicating a general tendency of mice to move at lower speeds under this experimental condition. The 3, 5, and 10 targets stimulation conditions show rightward shifted cumulative distributions, suggesting that mice moved faster under these other experimental settings. Although all mice were habituated to the running wheel and the setup before experiments started, one possibility is that as experimental sessions progressed (stimulation of 1 target was conducted first and stimulation with 10 targets was conducted last), mice became more comfortable on the wheel and therefore moved at a faster speed (see also section 5 for further discussion of this point). Inspection of the distributions of photostimulation trials occurring in periods of quiet wakefulness (Q) and locomotion (L, Figure 22D, E) confirmed that experiments in which 1 target was stimulated were characterized by only a minority of photostimulation events occurring during locomotion periods, with uneven distribution also across animals, experiments, and locomotion speed (Figure 22B, E).

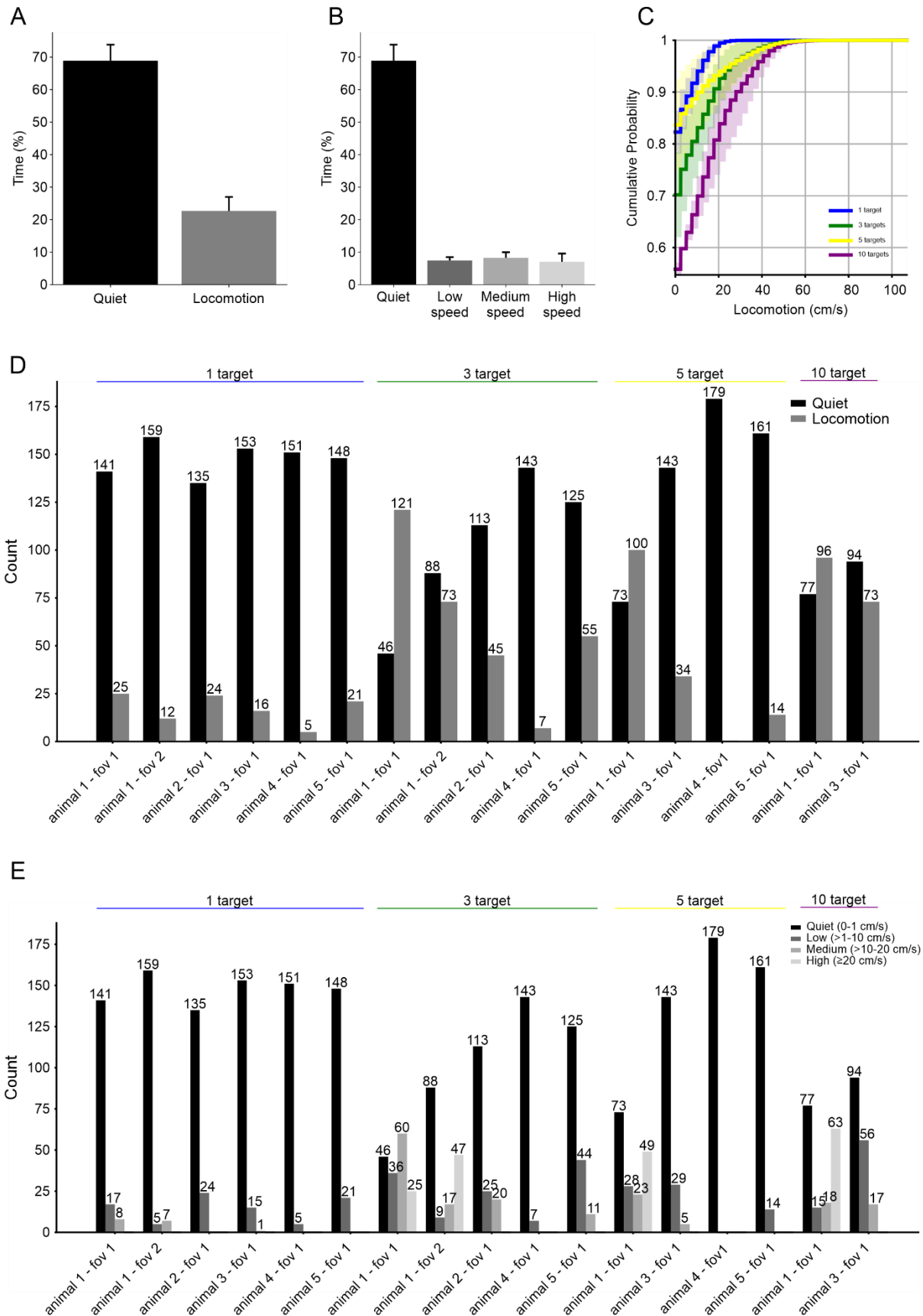


Figure 22. Locomotion behavior during experimental sessions

A. Percentage of time spent by all mice ($n = 5$) in quiet (Q) vs locomotion (L) states across experimental sessions. Values are represented as mean $\pm$ SD. **B.** Percentage of time spent by all mice ($n = 5$) in quiet vs locomotion states, with locomotion states categorized based on mean velocities (see main text for details). Values are represented as mean $\pm$ SD. **C.** Cumulative speed distributions across mice. The color code indicates stimulation conditions. Values are represented as mean $\pm$ SEM. **D.** Number of photostimulation trials occurring during Quiet wakefulness (black) and Locomotion (dark grey) for each experimental session. **E.** Same as (D), but trials occurring during Locomotion are further subdivided based on the speed of the animal (Low speed, dark grey; medium speed, grey; high speed, light grey).

We started off examining photostimulation responses of target neurons and non-target neurons, without accounting for behavioral state or the number of targeted neurons. As expected, target cells displayed significantly higher responses compared to non-targets (Figure 23).

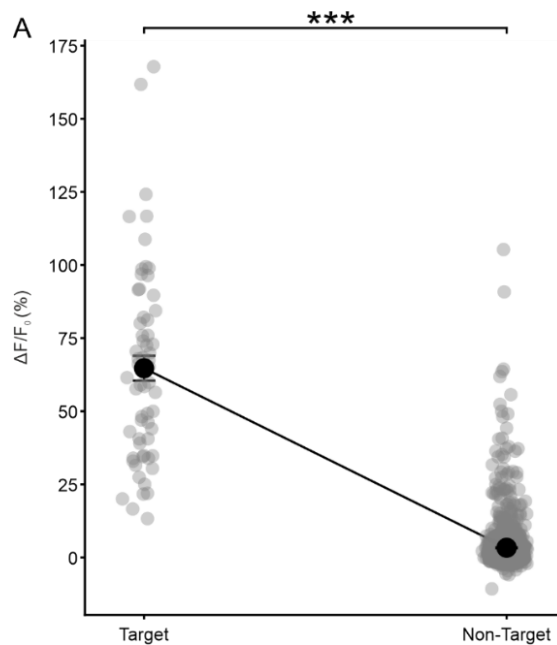


Figure 23. Target and non-target neurons mean responses to photostimulation

A. Mean responses ($\pm$ SEM) to photostimulation of targets (mean = 0.64 ± 0.04 , $N = 61$) and non-target cells (mean = 0.033 ± 0.001 , $N = 1653$). Mann-Whitney U test ($p = 3.07e-39$).

When locomotion was taken into account, we observed that target neurons did not show a significant difference between the Q and L states (Figure 24A). Non-target cells, instead, displayed a small but significant decrease in average activity during photostimulation of targets in the L state. We also noted a broader distribution of responses during locomotion compared to the Q state (Figure 24B).

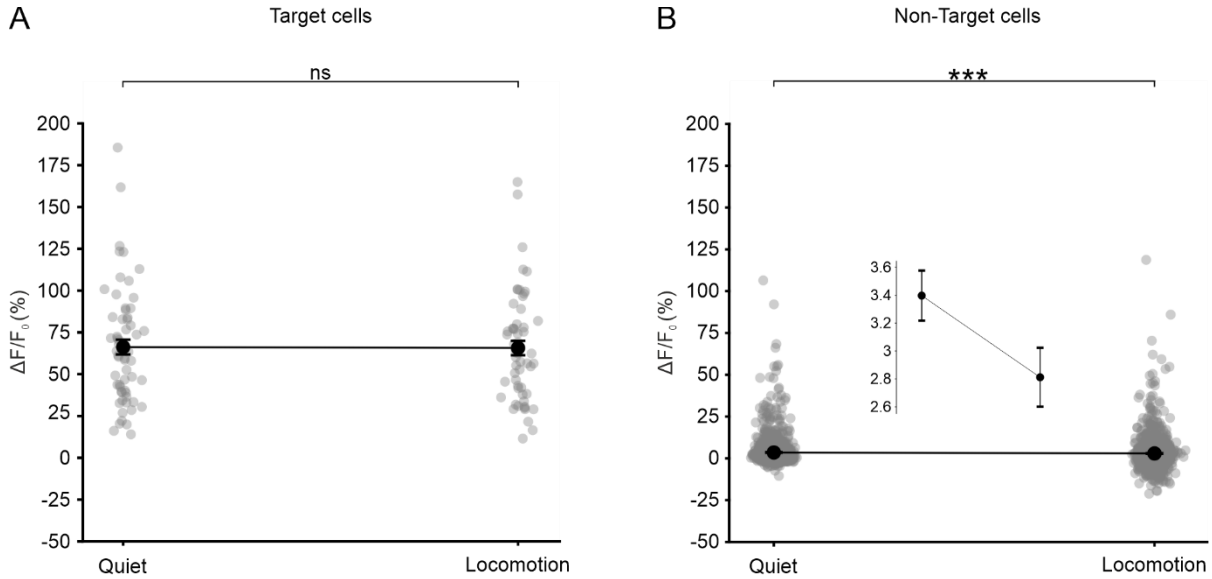


Figure 24. Response of target and non-target neurons to photostimulation during quiet and locomotion states

A. Mean responses ($\pm$ SEM) to photostimulation of target cells in the Q (mean = 0.66 ± 0.04 , $N = 61$) and L states (mean = 0.65 ± 0.04 , $N = 56$). Wilcoxon signed rank test ($p = 5.83 \cdot 10^{-2}$). **B.** Same as in (A) but for non-target cells (Q state: mean = 0.034 ± 0.001 , $N = 1653$; L state: mean = 0.028 ± 0.002 , $N = 1573$. Wilcoxon signed rank test ($p = 2.62 \cdot 10^{-5}$).

This first analysis indicated that locomotion influenced the response of non-target cells, with a small average decrease in responses upon locomotion. To better understand whether the activation of target cells was exerting an excitatory, neutral, or inhibitory influence on non-target neurons, we computed an “influence measure” for each non-target neuron (see section 3.8; Khoury et al., 2025), such that excited neurons would be those whose mean activity significantly increased in correspondence with photostimulation, inhibited neurons those

whose mean activity significantly decreased in correspondence with photostimulation, and neutral neurons the remaining ones. We first computed the influence score without accounting for locomotion and the number of target cells (Figure 25).

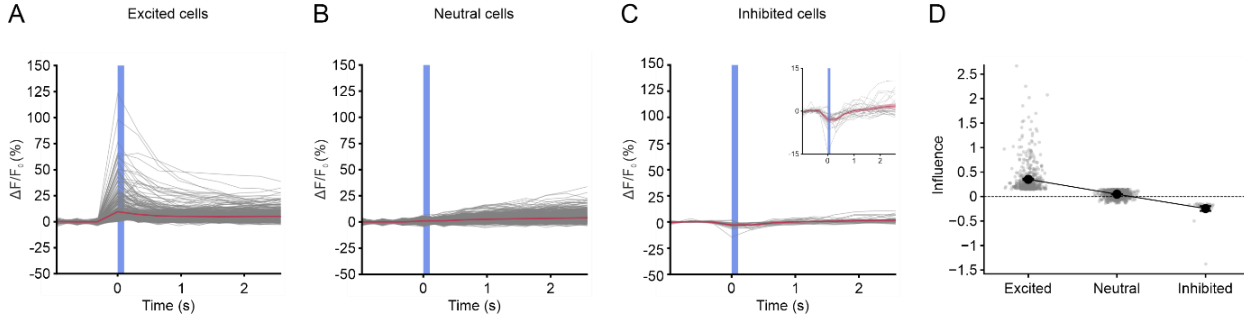


Figure 25. Excited, neutral, and inhibited network neurons after photostimulation

A. Stimulus-triggered averages of excited non-target neurons. Average responses from single cells (grey) and mean activity across cells $\pm$ SEM (red) are shown. Blue bar indicates photostimulation time interval. **B.** Same as in **A**, but for neutral non-target neurons. **C.** Same as in **A**, **B**, but for inhibited non-target neurons. **D.** Average influence scores for excited, neutral, and inhibited non-target neurons $\pm$ SEM. Excited cell, $N = 538$; neutral cells, $N = 1090$; inhibited cells, $N = 25$.

The majority of non-target cells (66% neutral cells; Figure 25A, D) remained unaffected by photostimulation of target neurons, almost one third of all cells (32.5% excited cells; Figure 25B, D) were excited, and a minor portion of neurons (1.5% inhibited cells; Figure 25C, D) was inhibited. We next refined our analysis and examined how the influence score in three functional classes (excited, neutral, and inhibited) would change as a function of behavioral state. We found that excited neurons were more excited during locomotion (Figure 26A), but fewer in absolute number (446 and 234 cells in Q and L states, respectively; Binomial test, $p < 0.05$). Inhibited neurons were both more inhibited and showed a non-significant variation in number (Figure 26C; 19 and 34 cells in Q and L states, respectively; Binomial test, $p > 0.05$). Neutral neurons did not display any significant difference in their influence score but presented a higher numerosity during locomotion (1188 and 1305 cells in Q and L states, respectively;

Binomial test, $p < 0.05$). Neutral cells also showed larger variability of the influence score values, similarly to what was observed for average responses (Figure 24B and Figure 26B).

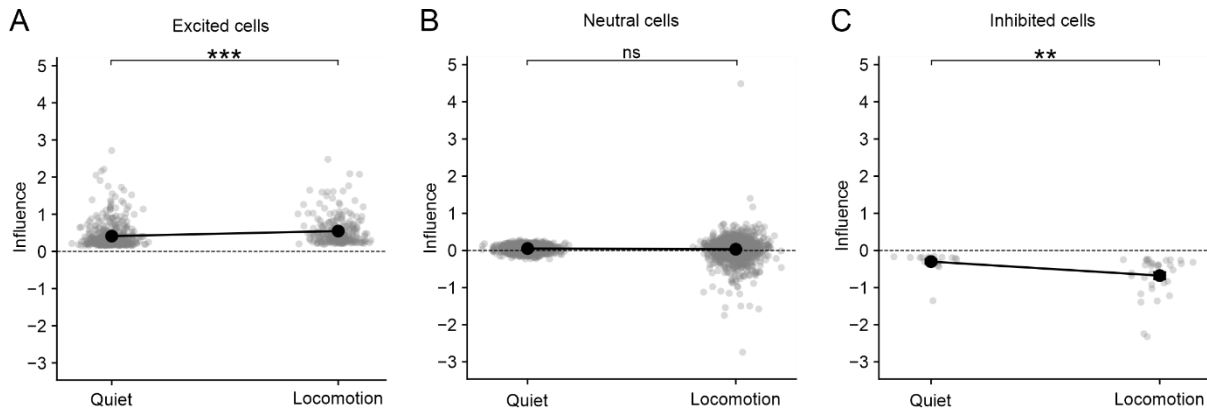


Figure 26. Excited, neutral, and inhibited network neurons after photostimulation during quiet and locomotion states

A. Influence score values for excited cells (grey) and average influence score across cells $\pm$ SEM (black) in Q (0.40 ± 0.01 ; $n = 446$) vs L (0.54 ± 0.02 ; $n = 234$) states. Wilcoxon signed rank test ($p = .$). **B.** Same as in (A) but for neutral cells in Q (0.05 ± 0.002 ; $n = 1188$) vs L (0.03 ± 0.008 ; $n = 1305$) states. Wilcoxon signed rank test ($p = 0.49$). **C.** Same as in (A) but for inhibited cells in Q (-0.03 ± 0.05 ; $n = 19$) vs L (-0.6 ± 0.08 ; $n = 34$) states. Wilcoxon signed rank test ($p = 0.0011$).

Next, we examined whether responses of target and non-target neurons differed based on the number of target cells. Initially, we did so regardless of locomotion (Figure 27).

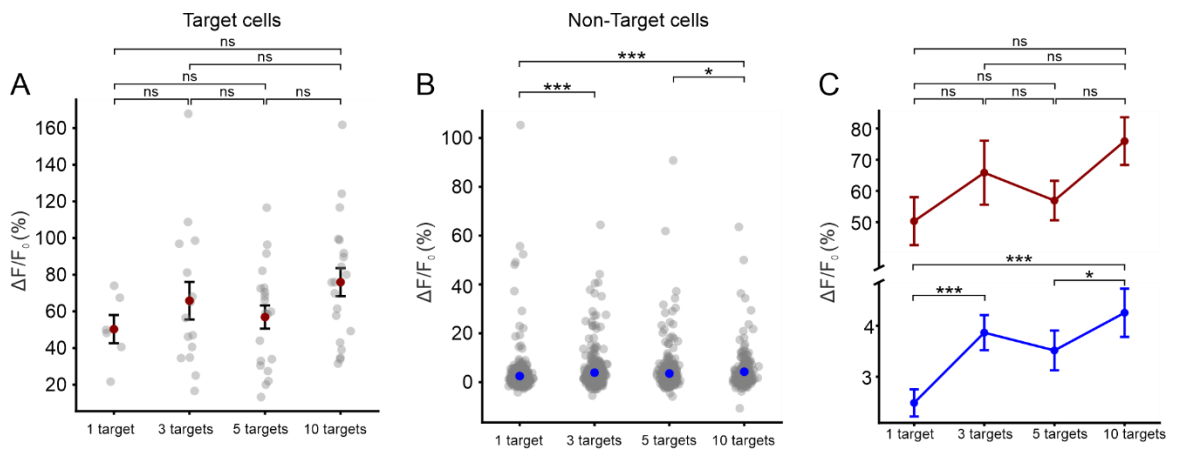


Figure 27. Mean responses of target and non-target neurons to photostimulation

A. Target cells mean responses to photostimulation (grey dots) and average response magnitude $\pm$ SEM across targets for each condition (black). Kruskal-Wallis test ($p = 0.21$). $n = 6, 15, 20, 20$ for the 1, 3, 5, and 10 targets conditions, respectively. **B.** Same as in (A), but for non-target cells. Kruskal-Wallis test ($p = 2.97e-7$), Dunn's test with Bonferroni correction for multiple comparisons. 1 target vs 3 targets, $p = 2.68e-4$; 1 target vs 10 targets, $p = 1e-6$; 5 targets vs 10 targets, $p = 0.03$. $n = 610, 423, 382, 238$ cells for the 1, 3, 5, and 10 targets conditions, respectively. **C.** Target (dark red) and non-target (blue) neurons' mean responses at an expanded y scale. All panels show mean $\pm$ SEM.

We confirmed that target cells displayed higher magnitude responses compared to non-target cells also when considering each stimulation condition in isolation (Mann-Whitney test with Bonferroni correction for multiple comparisons. 1 target, $p = 7.3e-11$; 3 targets, $p = 9.3e-11$; 5 targets, $p = 1.6e-13$; 10 targets, $p = 1.8e-13$), in agreement with the fact that target cells are directly stimulated (Figure 23A, B). Given the small but significant difference in the response magnitude of non-target neurons between the 1 target and the 3 and 10 targets conditions, one possibility is that the differences in pre-synaptic activity imposed with photostimulation transfer to non-target neurons. However, it is also possible that the effect we observe on non-target neurons is biased by higher speeds of mice observed under the different experimental conditions (Figure 22C, E).

We checked if, by further classifying photostimulation trials as occurring during periods of quietness or locomotion, we could observe any difference in the neuronal responses to photostimulation. Again, target cells were not affected by locomotion ($p > 0.013$ for all comparisons; Figure 28A), while non-target cells showed a progressive decrease in their response magnitude as a function of the number of targeted cells in the locomotion state compared to the quiet state (Figure 28B). This observation resulted statistically significant for the 5 and 10 targets photostimulation conditions.

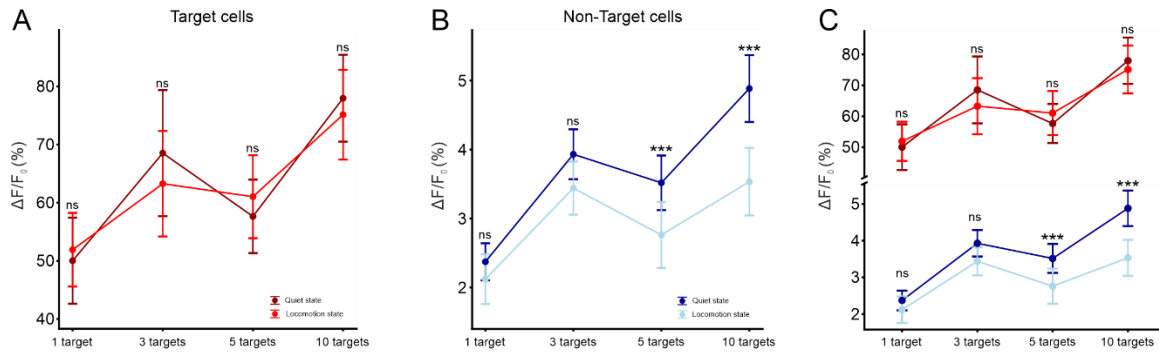


Figure 28. Target and non-target neurons mean responses to photostimulation during quietness and locomotion

A. Average response magnitude $\pm$ SEM of target cells for each condition when the mouse was still (Q, dark red) or locomoting (L, red). Wilcoxon signed rank test with Bonferroni correction for multiple comparisons ($p > 0.0125$ in all comparisons). $n = 6, 15, 20$ (Q)/ 15 (L), 20 cells for the 1, 3, 5, and 10 targets conditions, respectively. The 5 targets condition has unequal sample sizes because one experimental session lacked running behavior during photostimulation events. **B.** Same as in A, but for non-target cells (Q, blue; L, light-blue). Wilcoxon signed rank test, Bonferroni correction for multiple comparisons. 1 target, $p = 0.60$; 3 targets, $p = 0.11$; 5 targets, $p = 0.0004$; 10 targets, $p = 2.3e-9$. $n = 610, 423, 382$ (Q)/ 302 (L), 238 cells for the 1, 3, 5, and 10 targets conditions, respectively. The 5 targets condition has unequal sample sizes because one experimental session lacked running behavior during photostimulation events. **C.** Target and non-target neurons' mean responses in the Q and L states. All panels show means $\pm$ SEM.

The small difference observed in $\Delta F/F_0$ when photostimulation is performed in Q or L periods might be due to differences in the induced firing rate by the same optical stimulation due to different electrical properties of network cells in the two behavioral states (e.g., resting membrane potential (H Petersen et al., 2003)).

We then applied again the influence metric to evaluate the effect of targets photostimulation on network activity, classifying non-target neurons as excited, neutral, and inhibited in the different stimulation conditions (Figure 29). We observed that a large number of excited neurons emerged: 23.77% in the 1 target condition, 33.80% in the 3 targets condition, 37.17% in the 5 targets condition, 44.11% in the 10 targets condition. Neutral cells were, however, the

more numerous class: 75.24% in the 1 target condition, 64.06% in the 3 targets condition, 60.99% in the 5 targets condition, 54.62% in the 10 targets condition. In contrast, inhibited cells represented the less numerous class: 0.98% in the 1 target condition, 1.89% in the 3 targets condition, 1.83% in the 5 targets condition, 1.26% in the 10 targets condition. While these data show a gradual increase in excited cells as a function of number of photostimulated targets, none of these three categories presented statistically significant differences (Kruskal-Wallis test. Excited cells, $p = 0.1$; neutral cells, $p = 0.11$; inhibited cells, $p = 0.36$).

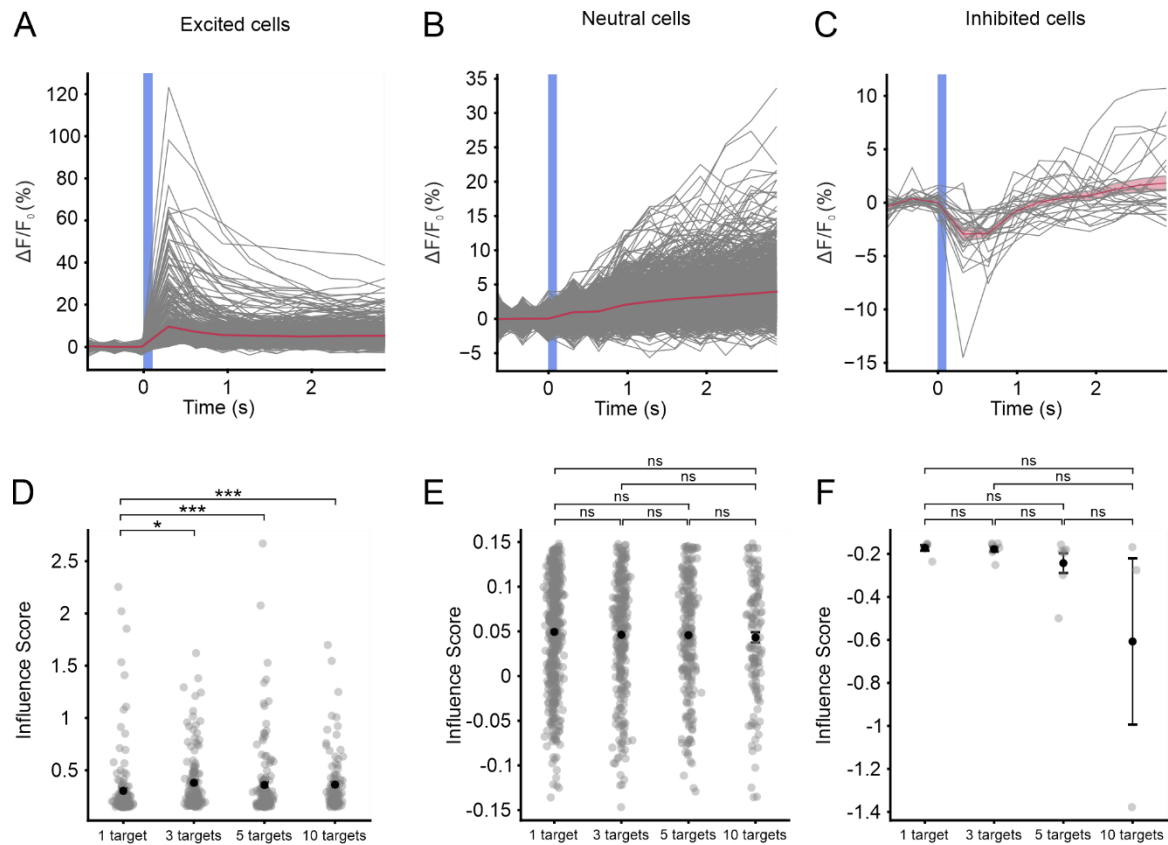


Figure 29. Photostimulation responses in non-target neurons: excited, neutral, and inhibited cells

A. Stimulus-triggered averages of excited neurons. Average responses from single cells (grey) and mean activity across cells $\pm$ SEM (red) are shown. Blue bar indicates photostimulation. **B.** Same as in **A** but for neutral network neurons. **C.** Same as in **A**, **B** but for inhibited network neurons. Average responses from single cells (grey) and mean activity across cells $\pm$ SEM (red) are shown. Blue bar indicates photostimulation of target neurons (excluded in this analysis). **D.** Influence scores for excited non-target neurons in the post-stimulation window grouped by condition. Kruskal-Wallis test ($p = 9.8758e-6$), Dunn's test with Bonferroni correction for multiple comparisons.

1 target vs 3 targets, $p = 1.77e-5$; 1 target vs 5 targets, $p = 0.04$; 1 target vs 10 targets, $p = 5.17e-4$. $n = 145, 143, 142, 105$ cells for the 1, 3, 5, and 10 targets conditions, respectively. **E, F.** Same as in **D**, but for neutral and inhibited network neurons, respectively. Kruskal-Wallis test on neutral neurons ($p = 0.74$). Kruskal-Wallis test for inhibited neurons ($p = 0.07$). Grey dots indicate average influence score across photostimulation trials for a specific cell. Black dots and bars indicate average influence score across cells $\pm$ SEM. Neutral cells: $n = 459, 271, 233, 130$ cells for the 1, 3, 5, and 10 targets conditions, respectively. Inhibited cells: $n = 6, 9, 7, 3$ cells for the 1, 3, 5, and 10 targets conditions, respectively.

Neutral and inhibited neurons, grouped regardless of locomotion, did not present statistically significant differences between conditions. Among excited non-target neurons, instead, the 1 target condition (mean influence score $\pm$ SEM = 0.11 ± 0.01 , $n = 145$) showed significant differences with all other three conditions (mean influence score $\pm$ SEM: 3 targets, 0.15 ± 0.01 , $n = 143$; 5 targets, 0.15 ± 0.01 , $n = 142$; 10 targets, 0.17 ± 0.02 , $n = 105$).

As previously done for average responses, we then subdivided stimulation trials based on whether they occurred during Q or L states and classified network neurons as excited, neutral, or inhibited in the two different states, this time accounting also for the number of photostimulated targets. We found that, in the Q state, the average influence measures of excited network cells replicated the same trend observed in Figures 27 and 28; neutral and inhibited cells, instead, presented different trends. In the Q state, excited neurons displayed a larger influence score than in the L state during the stimulation of 1 or 5 targets (Figure 30A). Neutral cells in the Q state display larger influence score when 10 targets were photostimulated compared to the L state (Figure 30B). Inhibited cells in the Q state displayed a smaller influence score compared to the L state.

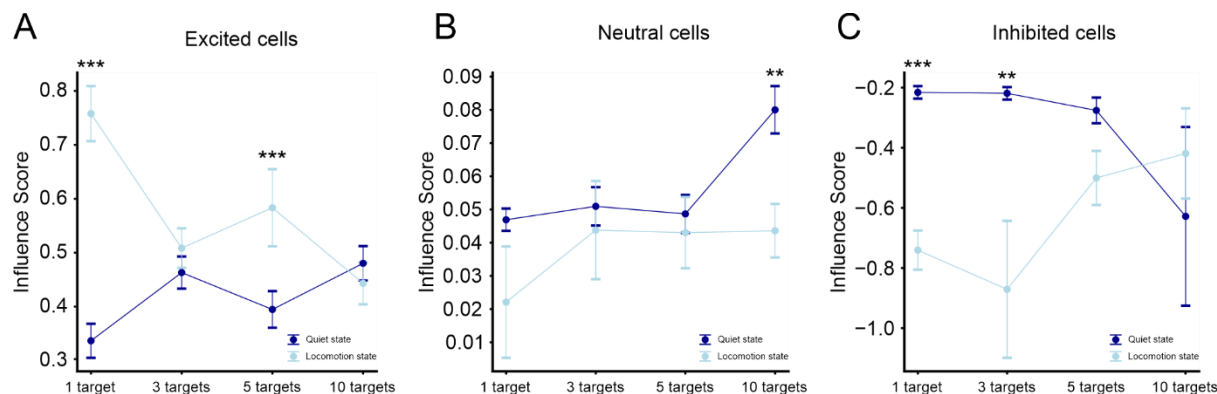


Figure 30. Excited, neutral, and inhibited non-target neurons during quiet and locomotion states

A. Influence score values for excited cells in Q vs L states. Mann–Whitney test with Bonferroni correction for multiple comparisons. 1 target, $p=8.75e-14$; 3 targets, $p = 0.06$; 5 targets, $p=1.80e-08$; 10 targets, $p = 0.12$. $n = 36, 87, 48, 64$ (L) and $127, 113, 126, 85$ (Q) for the 1, 3, 5, and 10 targets conditions, respectively. **B.** Influence values for neutral cells in Q vs L states. Mann–Whitney test with Bonferroni correction for multiple comparisons. 1 target, $p = 0.07$; 3 targets, $p = 0.19$; 5 targets, $p = 0.6$; 10 targets, $p=0.004$. $n = 565, 325, 247, 167$ (L) and $478, 306, 250, 150$ (Q) for the 1, 3, 5, and 10 targets conditions, respectively. **C.** Influence values for inhibited cells in Q vs L states. Mann–Whitney test, Bonferroni correction for multiple comparisons. 1 target, $p=9.99e-04$; 3 targets, $p=9.16e-04$; 5 targets, $p = 0.07$; 10 targets, $p = 0.83$. $n = 9, 11, 7, 7$ (L) and $5, 4, 6, 3$ (Q) for the 1, 3, 5, and 10 targets conditions, respectively. All values represent average across cells $\pm$ SEM.

At the time of writing, pupil recordings have not been analyzed yet. As such, the results described here take into account only the statistical correlation between locomotion and neuronal activity recordings.

4.4 Co-expression of RCaMP3 and stCoChR using a novel bicistronic construct

In the framework of a scientific collaboration between the Fellin research group and that of Professor M. Sakamoto, researchers from the University of Kyoto engineered a single adeno-associated virus (see section 3.2) containing the genes encoding for RCaMP3 (Yokoyama et

al., 2021) and stCoChR (Forli et al., 2021), separated by the P2A cleavage site. In this bicistronic vector, the indicator and opsin coding sequences were placed under the CaMK-II α promoter to restrict their co-expression to excitatory cells. We injected the virus containing the bicistronic construct into the primary somatosensory cortex of adult C57BL/6 mice (Figure 31B). Two months postinjection, RCaMP3 fluorescence was visible (N = 3 animals, imaged from 3 to 20 weeks postinjection), and we started performing all-optical 2P imaging and stimulation experiments.

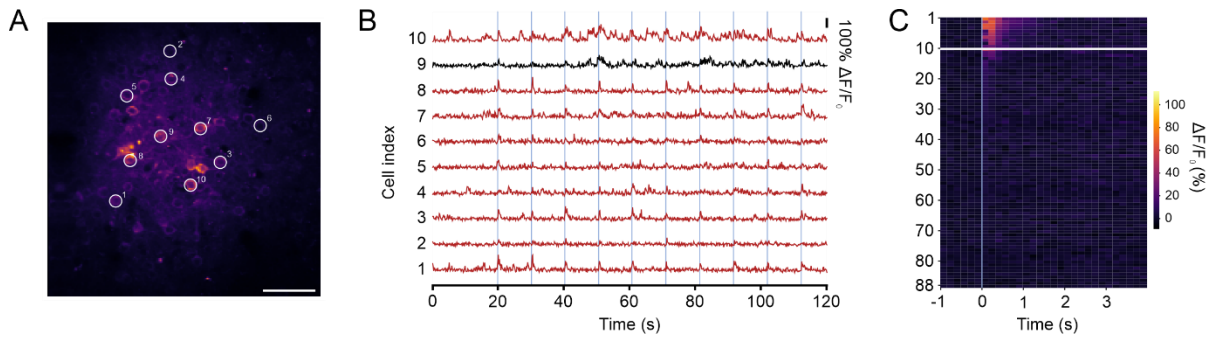


Figure 31. All-optical experiments in mice expressing RCaMP3 and stCoChR using a novel bicistronic construct.

A. Field of view showing excitatory neurons co-expressing RCaMP3 and stCoChR. Scale bar = 50 μm **B.** $\Delta F/F_0$ traces from the ten target neurons indicated in **A** with a white circle. Blue bars indicate the first 10 photostimulation events of the experiment. Red traces indicate the neuron was classified as responsive, black traces correspond to non-responsive neurons. Same stimulation protocol as in section 4.3. **C.** Raster plot showing average activity of all neurons recorded in the experiment in **(B)**, sorted by peak activity after photostimulation (blue bar). The white horizontal bar separates target from non-target neurons.

We tested photostimulation of multiple cells (8-10 targets per ensemble) to control for cell responsiveness in different fields of view in three mice freely running on a wheel. On a total of 991 recorded cells (123.88 ± 94.40 neurons per FOV, 8 FOVs), we tested 108 target neurons (Figure 32A), 53 (49.1%) of which resulted responsive to photostimulation (same definition of responsive neuron as that used in Figure 18). The average peak value among neurons classified

as responsive ($n = 53$) was $46.2 \pm 2.5\% \Delta F/F_0$. We then computed the influence score (see section 4.3 for definition) on non-target neurons of this new dataset. We observed 173 excited neurons, 705 neutral neurons, and 5 inhibited neurons, corresponding to 19.6% excited, 79.8% neutral, and 0.6% inhibited cells out of a total of 883 non-target neurons.

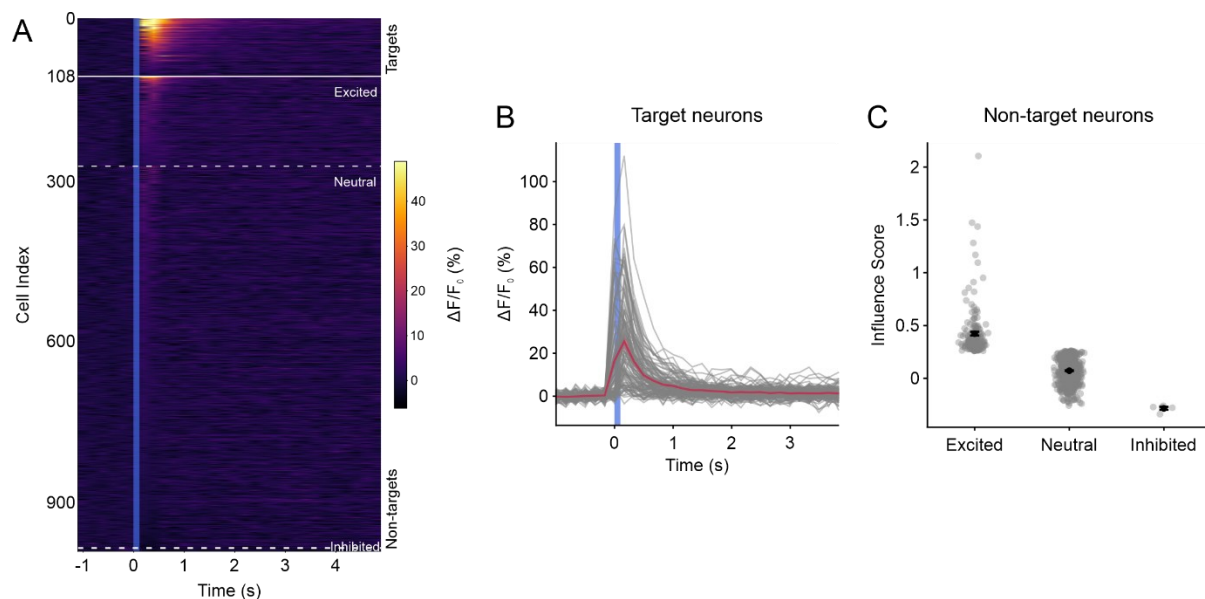


Figure 32. All optical 2P imaging and stimulation experiments in S1 principal neurons using a bicistronic viral vector

A. Raster plot showing average activity of all neurons sorted by peak activity after photostimulation (blue bar) recorded in 2 experimental sessions from 3 mice in correspondence of photostimulation (blue bar). The white horizontal bar separates target from non-target neurons. **B.** Stimulus-triggered average responses from all target neurons. Average activity of single neurons (grey) and average across all cells $\pm$ SEM (red) are shown. **C.** Influence scores for excited, neutral, and inhibited non-target neurons in the post-stimulation time window (see Materials and Methods for definition).

5. Discussion

Two-photon all-optical technologies enable simultaneous high-resolution two-photon imaging and perturbation of neuronal networks at the cellular level, representing a crucial tool to investigate the relationship between neural circuit and behavior (Adesnik & Abdeladim, 2021; Adesnik & Naka, 2018; Bovetti & Fellin, 2015; Carrillo-Reid et al., 2017; Carrillo-Reid & Yuste, 2020; Chen et al., 2018; Deisseroth, 2015; Emiliani et al., 2015; Emiliani & Ofer, 2022; Oron et al., 2012; Panzeri et al., 2017; Ronzitti et al., 2017, 2018; Russell et al., 2022; Vaziri & Emiliani, 2012; Yizhar et al., 2011). The two-photon all-optical toolkit has undergone significant improvements over the years, expanding the range of addressable questions by introducing both optical and molecular advancements. On the one hand, optical developments allowed to move from imaging and control over the activity of a few neurons in cell culture or brain slices (Lutz et al., 2008; Nikolenko et al., 2007, 2008; Dal Maschio et al., 2010), to awake behaving mice (e.g., Bounds et al., 2025; Buetfering et al., 2022; Carrillo-Reid et al., 2019; Daie et al., 2021; Dagleish et al., 2020; Gill et al., 2020; Packer et al., 2015; Rickgauer & Tank, 2009; Russell et al., 2024), over extended volumes and large field of views (Abdeladim et al., 2023; Dal Maschio et al., 2017; Pégard et al., 2017; Yang et al., 2018), and freely moving animals (Accanto et al., 2023). Additionally, several optical methods have been deployed to maximize the power budget under the microscope objective while minimizing the average power needed to excite individual neurons (Forli et al., 2021; Picot et al., 2018; Ronzitti et al., 2017; Yang et al., 2018). On the other hand, molecular tools also greatly improved, providing researchers with novel two-photon excitable calcium indicators and opsins (Akerboom et al., 2013; Chen et al., 2013; Dana et al., 2016, 2019; Yokoyama et al., 2024; Fink et al., 2024; Boyden et al., 2005; Klapoetke et al., 2014; Forli et al., 2018, 2021), optimizing their

expression levels (Chen et al., 2013; Dana et al., 2016; Lin et al., 2013; Marshel et al., 2019; Russell et al., 2022), increasing their conductance (Klapoetke et al., 2014; Forli et al., 2021; Vierock et al., 2021; Marshel et al., 2019; Sridharan et al., 2022), restricting their localization to somatic membranes (Baker et al., 2016; Chettih & Harvey, 2019; Forli et al., 2018, 2021; Lim et al., 2000), and developing new classes of indicators for direct accessibility to neuronal electrical (Adam et al., 2019; Knöpfel & Song, 2019, see sections 1.2.2 and 1.3.1) or neuromodulatory activity (Feng et al., 2019; Patriarchi et al., 2018; Sabatini & Tian, 2020; Sun et al., 2018; Wan et al., 2021). Nonetheless, current two-photon all-optical approaches still suffer from limitations. One such limitation is the crosstalk between imaging and photostimulation (Forli et al., 2018, 2021; Packer et al., 2015; Rickgauer et al., 2014; see also introduction). To address this limitation, experimental solutions based on the use of blue-shifted opsins and red-shifted calcium indicators have been developed (Forli et al., 2021; Fink et al., 2023). However, this strategy suffers from the traditionally low performance of red-shifted calcium indicators (Dana et al., 2016) compared to green-shifted indicators (Chen et al., 2013; Dana et al., 2019; Zhang et al., 2023). Recently, new promising red-shifted calcium indicators have been developed: RCaMP3 (Yokoyama et al., 2024) and PinkyCaMP (Fink et al., 2024).

In this thesis work, we combined one of these indicators, RCaMP3 (Yokoyama et al., 2024), with the blue-shifted large-conductance opsin stCoChR (Forli et al., 2021) to enable high-performance all-optical experiments with minimal crosstalk between imaging and photostimulation. First, we demonstrated that RCaMP3 displays a two-photon absorption spectrum (Figure 16) that is compatible with two-photon all-optical experiments in

combination with blue-light-sensitive opsins. Specifically, RCaMP3 can be excited at 1080-1100 nm, a wavelength range that is not efficient in stimulating stCoChR. RCaMP3 can be excited at 920 nm (Figure 16B), and this may potentially lead to unwanted artifacts caused by the excitation of RCaMP3 by the photostimulation laser. To control for this issue, we photostimulated cells expressing only RCaMP3 in vivo and found non-significant increases in median RCaMP3 fluorescence values between the pre-stimulation and stimulation windows. We then achieved co-expression of RCaMP3 and stCoChR initially using two independent viral vectors, obtaining unprecedented readout capabilities leveraging on the high-SNR RCaMP3 response combined with efficient two-photon excitation of stCoChR using a limited average power per cell, thereby minimizing tissue heating (Figure 18, 21). We employed this experimental preparation to investigate how neuronal responses to photostimulation are influenced by the animal behavioral state for both target and non-target neurons. To this aim, we repeatedly photostimulated target ensembles of various dimensions (number of targets, 1, 3, 5, 10 cells) while recording the pupil diameter and running speed of head-fixed mice. The analysis of average photostimulation responses during quiet and locomotion states for target and non-target neurons (Figure 24) revealed that target cell responses were not statistically affected by locomotion activity, while non-target cells displayed a small but significant decrease in average activity during locomotion periods. When accounting for the number of stimulated targets, the results were similar: target cells were never affected by locomotion, while non-target cells exhibited a progressive decrease in their response magnitude in the locomotion state compared to the quiet state as a function of the number of stimulated cells (Figure 28B).

Non-target cells response amplitudes seemed to depend, at least in part, on the number of stimulated target cells (Figure 27B and 28B). However, stimulation with different target cells number occurred in time windows characterized by different levels of locomotion (Figure 22C, E). Therefore, it is not possible to dissect out, in this dataset, whether the effect observed on the amplitude of photostimulation responses is due to the different number of stimulated target cells or to the different level of ongoing locomotion speed. Longer habituation times, randomization of stimulation conditions (i.e., the number of target neurons) across recording days, and larger statistical samples represent the next experimental step.

Overall, these results indicate that locomotion does exert an influence on non-target cells activity during photostimulation of target cells. In contrast, target cell response was not affected by locomotion. One possibility is that the low numerosity of the target cells population influenced the results of the statistical comparison (Figure 24A, 27A, 28A). Future experiments will be needed to increase the sample size of the target neuron sample.

Wheel running has been previously reported to hyperpolarize S1 layer 2/3 excitatory neurons, with running velocity being negatively correlated with membrane potential (Fisher et al., 2016; Shimaoka et al., 2018). Contrasting evidence has reported increased activity in S1 layer 2/3 principal cells during locomotion or whisking behavior, which in mice is closely associated with locomotion (Ayaz et al., 2019; Crochet & Petersen, 2006; Poulet & Petersen, 2008). In our dataset, we currently miss a quantification of the effect of locomotion on spontaneous network activity in the absence of photostimulation. We are planning new experiments in this direction which will help interpreting the results obtained so far.

Quantification of the influence score metric showed that excited non-target cells were more excited and inhibited cells were more inhibited during locomotion compared to quiet periods (Figure 26A, C). Moreover, excited and neutral cells showed a significant difference in the number of neurons classified as excited and neutral between the quiet and the locomoting states, with excited cells decreasing and neutral neurons increasing during locomotion. These results could be explained by locomotion-evoked increased network inhibition in S1 (Shimaoka et al., 2018). During locomotion, the absolute number of excited neurons could decrease relative to the Q state due to underlying inhibition. Cells with smaller excited response to photostimulation could be the most likely to show neutral photostimulation response during periods of increased inhibition, leading to increased numerosity of neutral cells during locomotion (Figure 26B). Future analyses are needed to track single cells identity and monitor potential switches between excited and neutral cell classes.

We next investigated the influence of the number of simultaneously stimulated target neurons on non-target cells. In our data, for the 5 and 10 targets conditions, we first observed lower average activity of non-target neurons during running compared to stationary periods, in correspondence to photostimulation events (Figure 28B). Importantly, differences between the quiet and the locomoting states were proportional to the number of photostimulated cells. If locomotion was hyperpolarizing the membrane potential of non-target cells, we could have expected that increasing the number of photostimulated cells would lead to a higher photostimulation-induced probability of firing, resulting in smaller, not larger, differences in average activity between the quiet and the locomoting states. However, it is also possible that, in an inhibitory-dominated network as is the cortex during locomotion (Polack et al., 2013;

Poulet & Petersen, 2008; Vinck et al., 2015), as the number of target neurons increased, more recurrent inhibition was recruited, leading to reduced $\Delta F/F_0$. Conversely, if basal network activity and cell membrane potential increased with locomotion, it is possible that photostimulation brought the network from a more to a less active state, again, via recruitment of local inhibition. In this scenario, further increasing the number of target neurons could possibly lead to an exacerbation of this phenomenon, ultimately amplifying local inhibition and accounting for the observed differences.

Russell et al. (2024) reported that, in their 2P all-optical experiments, the average activity of non-targeted neurons was suppressed by photostimulation of targets, and increasing the number of targets increased the magnitude of the suppression on non-target cells. However, they did not observe differences between the "more engaged" (high arousal) and "less engaged" (low arousal) states. Recomputing the influence metric accounting also for the number of targets revealed that excited non-target neurons showed a significant difference in influence magnitude in the locomotion state at the 1 and 5 target conditions (Figure 30A), with cells being more excited during locomotion. Inhibited cells showed stronger inhibition in the locomotion state at the 1 and 3 targets conditions (Figure 30C). Both excited and inhibited cells seem to converge toward the same average influence magnitude at the 10 targets condition. Our data suggest, again, state-dependent modulation of neuronal response to photostimulation. Overall, our preliminary findings appear to contrast with the reports from Russell et al. (2024). It must be noted, however, that this discrepancy can have several explanations, such as the fact that locomoting behavior was not directly taken into account in the Russell et al. paper, the two studies employed different sensory areas, and the inhibitory effect of photostimulation Russell et al. report was observed with higher numbers of targets. Indeed, another report (Rowland et

al., 2023) showed that photostimulation of large numbers of S1 neurons resulted in comparable fractions of excited and inhibited neurons in the local network, indicating that the local network maintained the excitation–inhibition ratio independently of stimulation strength (i.e., the number of target neurons). To identify the origin of the discrepancies between the studies cited above and our results, further experiments will be needed.

One frequent limitation of all optical two-photon imaging and photostimulation experiments regards the co-expression level of the opsin and the functional indicator. One strategy to achieve co-expression is to use two distinct viruses, as done in the first part of this thesis work, one carrying the construct encoding the opsin and the other one carrying the construct encoding the indicator. Several groups have optimized their co-expression strategies using two viral vectors by carefully calibrating viral titers and volumes (Russell et al., 2022; Chettih & Harvey, 2019; Dalglish et al., 2020; Marshel et al., 2019; Packer et al., 2015). However, co-expression with two viruses frequently results in a low number of co-expressing cells, limiting the applicability of the methods, especially for studies focusing on the functional role of dense ensembles of neurons. To address these limitations, we undertook a collaborative effort with Professor Sakamoto's group to generate and validate a new bicistronic viral construct (AAV2/1-CamKII-RCaMP3-P2A-stCoChR-WPRE), with the aim of enabling high-throughput functional co-expression of RCaMP3 and stCoChR in excitatory neurons. Using this novel bicistronic construct, we observed widespread expression of RCaMP3 at various cortical depths within the primary somatosensory cortex. However, in 2P all optical experiments, out of the 108 photostimulated target neurons, 53 (49.1%) responded, a lower fraction compared to other similar approaches (~80% with Ai203 transgenic mouse line,

Bounds et al., 2022; ~80% with bicistronic viral vector expressing jGCaMP8s – ChrimsonR, LaFosse et al., 2023; ~90% with ChRmine, ~85% with ChroME2s, ~75% ChroME2f, Sridharan et al., 2022), and to our dual viral approach (Figure 18). To explain our observation, one possibility is that the viral expression of a bicistronic virus under the CaMK α II leads to moderate expression levels of both the indicator and the opsin. It must also be considered that the photostimulation protocol we used to quantify the number of responsive targets in the dual viral approach (Figure 18) employed higher power per cell and 5 times longer illumination duration compared to the one used with the bicistronic vector (7 mW per target, 500 ms duration and 5 mW per target, 100 ms duration for the dual virus approach and for the bicistronic virus approach, respectively). These considerations would explain the lower number of responsive neurons to photostimulation compared to other studies using bicistronic viruses employing different promoters (e.g., LaFosse et al., 2023) and compared to our dual viral approach, as well as the lower average fluorescence amplitude of responsive neurons compared to the values observed in responsive neurons infected by two separate viruses. Interestingly, preliminary inspection of the data indicates that the lower response amplitudes observed in bicistronic-expressing cells classified as responsive resemble the amplitudes of spontaneous activity transients (Figure 31B). This seemed not be the case for the dual virus preparations. It is therefore possible that expression levels and stimulation conditions employed in the case of the bicistronic construct more effectively recapitulate the physiological range of spiking activity. A detailed characterization of the elicited spiking activity via cell-attached electrophysiological recordings of bicistronic-expressing cells and simultaneous imaging and photostimulation is planned to address this open point. In any case, the bicistronic viral approach will grant high quantities of functionally co-expressing neurons.

Altogether, these results demonstrate that the high-SNR, red-shifted indicator RCaMP3 can be co-expressed with the large-conductance, blue-shifted opsin stCoChR, enabling high-efficiency all-optical interrogation of neuronal circuits in vivo with effective spectral separation between the excitation spectra of the opsin and that of the calcium indicator, while maintaining minimal average stimulation power per cell. Preliminary analysis of experiments employing this preparation to investigate the influence of arousal on the two-photon photostimulation effect suggests that locomotion does exert an influence on how local neuronal networks respond to optogenetic activation (Figure 23B and 26). It is possible that the number of photostimulated cells exerts a modulatory effect on this phenomenon (Figures 22B, C; 23B; 24D; 26), but further experiments are needed to draw a firm conclusion on this point. Finally, the novel bicistronic viral vector characterized in this thesis work enables the efficient co-expression of RCaMP3 and stCoChR, maximizing the number of potential target neurons in all-optical preparations while granting minimized optical crosstalk. This viral strategy will likely play an important role in future all-optical studies.

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