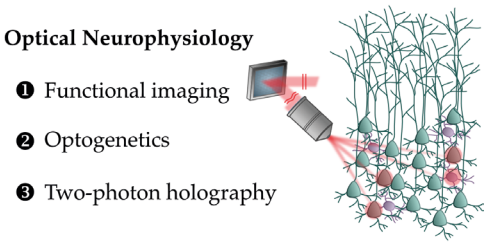
# ALL-OPTICAL NEUROPHYSIOLOGY WITH HOLOGRAPHIC LIGHT SHAPING

**Christiane GRIMM and Valentina EMILIANI***
Institute de la Vision, Sorbonne Université, INSERM, CNRS, Paris, France
***valentina.emiliani@inserm.fr**




https://doi.org/10.1051/photon/202513426



**To unravel how the mammalian brain computes in health and disease, neuroscientists need techniques that can non-invasively interrogate and manipulate neuronal activity with high spatiotemporal precision. In recent years, new approaches have emerged to achieve this goal by combining advanced light-shaping optical methods, two-photon excitation, and genetic tools for calcium, voltage imaging and optogenetics. Here, we provide a brief overview of these approaches, which lay the foundation for fully optical neurophysiology in both head-restrained and freely moving animals.**

## OPTICAL NEUROPHYSIOLOGY

Understanding brain function requires the investigation of neuronal circuitry across multiple spatiotemporal scales with the goal to map circuit activity and identify the respective behavioral correlates. One fundamental question is how these circuits operate: How are neurons connected within the circuit and to other circuits? Are individual neurons equivalent or do they form hierarchically organized assemblies? Do certain cells act as hubs orchestrating circuit activity and to what extent is a given spatiotemporal firing pattern necessary and unique for a particular behavior?

Answering these questions requires the ability to observe and also manipulate circuit activity with single-cell and single spike precision. Electrophysiological approaches can provide single-cell resolution when using intracellular electrodes, or population-level information when employing multi-electrode arrays and have greatly advanced the understanding of circuit dynamics to date. However, recordings at cellular resolution are low in throughput while population recordings lack single-cell resolution. In general, electrical techniques offer no access to the genetic identity of the recorded cells and are inherently invasive. In the case of intracellular recordings, this contact is even more disruptive and not compatible with longitudinal studies that require stable recordings over extended periods.

To overcome the limitations of electrical recordings, a collaborative effort among molecular biologists, physicists, biophysicists, and chemists has aimed to replace electrodes with light, both to read and manipulate electrical activity non-invasively, holding high potential especially for neuroscience applications. This interdisciplinary effort has given rise to the field of all-optical neurophysiology [1], which combines the genetic specificity of molecular tools with the

spatial and temporal precision of optical techniques.

## READING NEURONAL ACTIVITY WITH LIGHT

The challenge of reading neuronal activity with light was initially addressed through the development of calcium indicators, and more recently, genetically-encoded calcium indicators (GECIs). GECIs exploit the fact that neuronal activity is accompanied by transient increases in intracellular calcium, which changes its fluorescence in response to calcium binding providing an indirect yet robust optical readout of neuronal activity. As genetic tools GECIs provide both cell-type specificity and compatibility with chronic imaging, making them powerful tools for monitoring population dynamics over extended periods.

The use of wide-field single photon illumination allows calcium activity to be imaged across large brain regions and, combined with confocal detection schemes, enables cellular resolution in superficial layers. However, achieving population imaging with single-cell resolution deep within scattering tissue requires the use of two-photon (2P) microscopy, a technique based on the principle of Ω absorption first predicted by Maria Goeppert Mayer, who was later awarded the Nobel Prize in Physics for her pioneering contributions to quantum mechanics.

While calcium indicators have revolutionized our ability to image neuronal activity, their relatively slow kinetics limit temporal precision, since calcium transients integrate electrical events over hundreds of milliseconds. To overcome this limitation, more recently genetically-encoded voltage indicators (GEVIs) have been developed to directly report membrane potential changes rather than relying on secondary calcium signals. Depending on the molecular design of the GEVI, electrical changes can be read out as variations in fluorescence intensity, emission wavelength, or fluorescence lifetime. When combined with fast optical modalities, like wide field imaging, two-photon random-access scanning, light-sheet, or holographic imaging, GEVIs provide a direct way to monitor the electrical dynamics of neuronal populations at both single-cell and network levels in real time.

Together, calcium and voltage indicators form the foundation of optical neurophysiology, offering a powerful, genetically targetable, and minimally invasive way to observe how neurons communicate and process information across the brain.

## CONTROLLING NEURONAL ACTIVITY WITH LIGHT

The ability to manipulate neuronal activity is provided by optogenetics, an emerging field that began with the discovery of genes encoding microbial light-sensitive ion transporters like Channelrhodopsin and Halorhodopsin. Expressed in neuronal cells these opsins directly convert absorbed photons into ionic currents, allowing activation or inhibition of neuronal firing with high spatiotemporal precision. Optogenetics has transformed neuroscience, offering an unprecedented, non-invasive way to perturb and control brain function paving the way to dissect neural circuitry. The majority of todays' optogenetic experiments have used relatively simple illumination methods with visible light used to illuminate large brain regions while genetically restricting expression to certain cell types. Due to that, wide-field illumination can only synchronously activate entire populations of neurons providing rather unphysiological perturbations, while dissecting neural circuitry and contributions of individual neurons requires the development of optical methods capable of illuminating individual or several cells independently.

2P scanning microscopy approaches largely adopted for population calcium imaging cannot

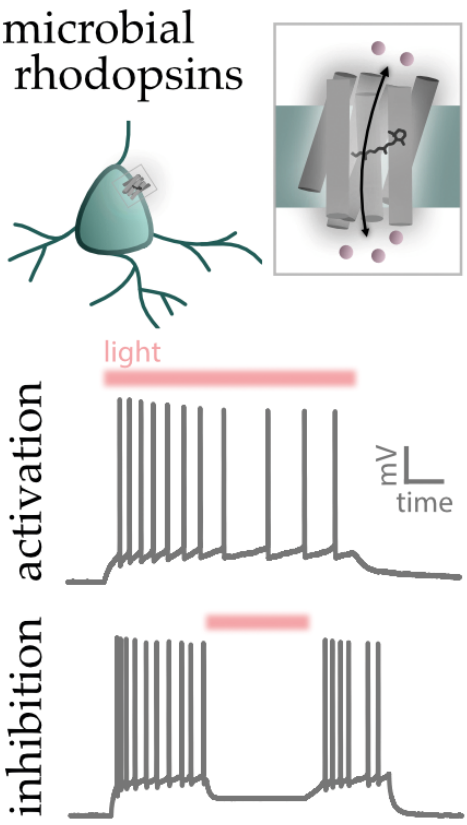



**Figure 1.** Microbial rhodopsins can be light-activated ion transporters that render ionic fluxes controllable with light. Genetically targeted to neuronal cells they allow control of neuronal activity with the spatiotemporal precision of the respective illuminaiton.

be directly translated to single-cell optogenetic stimulation for two main reasons. First, the diffraction-limited focal spot employed in conventional scanning microscopy is too small to illuminate the entire soma and efficiently recruit a sufficient number of opsin molecules to reliably trigger an action potential or maintain inhibition. Second, the temporal constraints imposed by sequential scanning hinder a true-parallel control of multiple target cells simultaneously.

## SCANLESS HOLOGRAPHIC ILLUMINATION

Some limitations of point scanning methods can be overcome through the use of holography, an ●●●

optical method originally introduced by Dennis Gabor (Nobel Prize in Physics, 1971). Holography works by modulating the wavefront of a laser beam so that the light field in the focal plane reproduces a user-defined spatial pattern. The modern implementation of this technique, known as Computer-Generated Holography (CGH) [2], relies on Spatial Light Modulators (SLMs) that can dynamically shape the phase of the incident beam. By computing holograms that correspond to specific positions, CGH allows the creation of multiple focal spots or customized illumination patterns within a 3D volume [3], each capable of stimulating individual neurons or neuronal ensembles.

When combined with 2P excitation, high-power pulsed lasers, and targeting strategies that restrict opsin expression preferentially to the neuronal soma, these approaches enable deep-tissue, parallel, and cell-specific optogenetic control, a powerful combination we refer to as circuit *optogenetics* [4].

Using CGH to create larger illumination areas covering the entire soma typically leads to a loss of axial resolution, which scales proportionally with the lateral spot size. To overcome this limitation, CGH can be combined with a technique known as temporal focusing (TF) [5], where ultrashort laser pulses are spectrally dispersed before being recombined at the focal plane. Because the pulse duration, and therefore the photon density, is restored only at the focal plane, excitation remains both temporally and spatially confined and allows the creation of extended excitation discs that uniformly illuminate the entire cell body while maintaining axial resolution comparable to that of a single diffraction-limited spot.

The combination of CGH and TF thus enables precise, parallel, and volumetric stimulation of multiple neurons in three dimensions, with subcellular spatial accuracy and millisecond temporal precision. After the demonstration of the first system for holographic 2P light patterning combined with TF, multiple variants have been developed to further enhance performance. These include approaches to reduce holographic speckle using generalized phase contrast, to extend the generation of temporally focused shapes in 3D, and to project light patterns at kilohertz rates, enabling sub-millisecond control of relative spike timing and increasing the number of targetable cells.

More recently, holographic light shaping has emerged as a key strategy for fast, multi-target 2P voltage imaging, allowing parallel, high-speed recording of voltage signals to detect neuronal activity with high contrast across multiple neurons [6]. This recent achievement underlines that holographic 2P illumination allows both 2P optogenetics and 2P voltage imaging rendering it an ideal illumination modality to set up all-optical neurophysiology approaches.

## ALL-OPTICAL NEUROPHYSIOLOGY

To date, most demonstrations of circuits optogenetics have been performed in head-restrained mice, where both imaging and photostimulation beams are delivered through a high–numerical-aperture objective via a cranial window. In these preparations, animals can be either anesthetized or awake allowed to run on a spherical treadmill or a rotating wheel, while their neuronal activity is simultaneously monitored and manipulated.

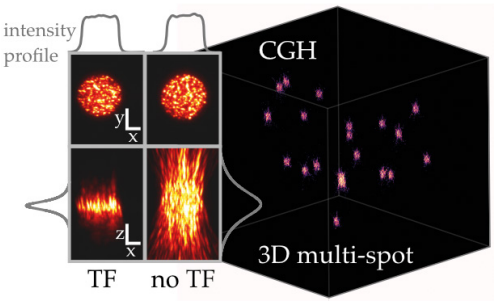



**Figure 2.** Three dimensional 2P holography for for targeting multiple cells with soma-sized spots. Inset showing intensity profiles of TF *vs.* No-TF spots.

Such experiments have successfully linked the activity of defined neuronal populations to sensory processing, motor control, and decision-making behaviors, providing direct evidence for the causal role of specific cells and circuits in brain function. These studies illustrate that precise spatiotemporal control of neuronal activity, enabled by techniques such as 2P holographic optogenetics, can reproduce physiologically relevant patterns of network activity, allowing researchers not only to observe but also to manipulate the flow of information through brain circuits.

However, many brain circuits, especially those involved in spatial navigation, social interaction, or goal-directed behavior, operate under naturalistic conditions that require freely moving animals. In recent years, 2P miniscopes have emerged as an extremely powerful approach for imaging calcium activity in freely moving mice or rats. In these devices, the scanning head used in conventional 2P microscopy is miniaturized to a size compatible with the animal's head, typically weighing less than 3 grams.

An alternative to miniaturized microscopes is to leave the complexity of the optical system on the optical table and use only a fiber and a focusing element, such as a GRIN lens or a mini-objective, as a relay between the output of the optical system and the animal's head *via* an adapter weighing less than 1 gram. To transmit holographic excitation patterns, the fibers used must belong to the class of fiber bundles composed of 15,000 to 30,000 individual multimode fibers allowing the holographic pattern to be faithfully transmitted to the output, which is then focused

onto the brain through the GRIN lens or mini-objective.

Following an initial demonstration of a holographic fiberscope in 2014, which used 1P holographic excitation combined with multi-confocal calcium imaging, a new class of endoscopes, called two-photon 2P-FENDO [7], has been recently developed using 2P excitation. An important property of the fibers used in 2P-FENDO is the inter-core differential delay, designed to reduce inter-core coupling. In the context of 2P excitation, this has the consequence that light injected into multiple cores simultaneously at the fiber entrance emerges at the output with temporal delays on the order of a few picoseconds per meter. As a result, the light produced at the focal plane of the GRIN lens or mini-objective does not form a single, uniform illumination spot. Instead, it appears as a random distribution of small bright points, each corresponding to an individual fiber core. This scattering effectively produces an illumination area equivalent to what would be obtained using a single illuminated core. Because the distribution of these points is sufficiently sparse, the axial resolution remains that of a single core, regardless of the number of cores illuminated providing intrinsic axial confinement without the need for temporal focusing.

Making advantage of this properties 2P-FENDO enable fast calcium imaging and single-cell optogenetic stimulation, enabling all optical experiments in freely moving animals at unprecedented spatiotemporal precision.

## CONCLUSION

The convergence of advances in genetically encoded indicators, optogenetic actuators, and holographic light-shaping approaches is making it possible to map, manipulate, and decode neuronal circuits across scales, with cellular resolution and millisecond temporal precision, thereby revealing causal links between activity patterns and behavior and giving rise to the field of all-optical neurophysiology. Recent progress in the development of genetically encoded voltage indicators and illumination methods compatible with two-photon voltage imaging has further enriched the field, enabling the readout of neuronal activity with single-spike resolution. Finally, as developments in fiber-based two-photon systems extend these capabilities to freely moving animals, optical neurophysiology is entering a new era in which complex, naturalistic behaviors can be studied with unprecedented resolution and specificity. Ultimately, these approaches are paving the way toward a mechanistic understanding of how distributed neuronal networks give rise to perception, cognition, and action.

## REFERENCES


[1] V. Emiliani, A. E. Cohen, K. Deisseroth, M. Hausser, J. Neurosci. **35**, 13917 (2015)

[2] J. Liesener, M. Reicherter, T. Haist, H. J. Tiziani, Opt. Commun. **185**, 77 (2000)

[3] N. Accanto, C. Molinier, D. Tanese, E. Ronzitti, Z. L. Newman, C. Wyart, E. Isacoff, E. Papagiakoumou, V. Emiliani, Optica **5**, 1478 (2018)

[4] I.-W. Chen, C. Y. Chan, P. Navarro, V. De Sars, E. Ronzitti, K. Oweiss, D. Tanese, V. Emiliani, Nat. Neurosci. **28**, 2141 (2025)

[5] E. Papagiakoumou, E. Ronzitti, V. Emiliani, Nat. Methods **17**, 571 ( 2020)

[6] R. R. Sims, I. Bendifallah, C. Grimm, A. S. M. Lafirdeen, S. Domínguez, C. Y. Chan, X. Lu, B. C. Forget, F. St-Pierre, E. Papagiakoumou *et al.*, Nat. Commun. **15**, 5095 (2024)

[7] N. Accanto, F. G. C. Blot, A. Lorca-Cámara, V. Zampini, F. Bui, C. Tourain, N. Badt, O. Katz, V. Emiliani, Neuron **111**, 176 (2023)