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Rethinking two-photon voltage imaging

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In this issue of *Neuron*, Grimm et al. describe the Jarvis voltage indicator, which demonstrates that scanless parallel two-photon excitation enables robust, high-speed voltage imaging *in vivo*, overturning prior assumptions about the incompatibility of rhodopsin-based indicators with two-photon microscopy.

Fluorescent indicators have become indispensable tools in neuroscience, enabling optical access to neural activity with cell-type specificity and spatial resolution. Among these tools, genetically encoded voltage indicators (GEVIs) offer millisecond temporal resolution to capture action potentials, synaptic integration, and subthreshold dynamics. At the mechanistic level, voltage indicators rely on two broad strategies to couple membrane voltage to fluorescence. One class exploits electrochromism, exemplified by microbial rhodopsin-based indicators, in which voltage-driven shifts in retinal protonation modulate fluorescence either directly or through electrochromic Förster resonance energy transfer (FRET). These FRET-opsin indicators are notable for their brightness, speed, and sensitivity. A second class relies on voltage-dependent conformational changes in engineered voltage-sensing domains (VSDs) to modulate fluorescence (Figure 1A).[1] Despite their distinct molecular mechanisms, both classes have enabled *in vivo* voltage imaging across a range of experimental systems, establishing voltage imaging as a powerful complement to calcium-based approaches.

Two-photon excitation has become essential for extending voltage imaging to intact brain tissue because it reduces out-of-focus background and improves penetration in scattering media. Yet, in contrast to its transformative impact on calcium imaging, voltage imaging under two-photon conditions has proven unusually difficult.[3] Part of this challenge lies in instrumentation, as voltage imaging demands kilohertz-scale sampling that pushes the limits of conventional scanning microscopes, although recent advances in high-speed scanning and scanless approaches have begun to ease this constraint.[4,5] Equally important are power-budget considerations, since the photon flux required to resolve fast voltage transients approaches limits set by photobleaching, heating, and photodamage. Beyond these optical factors, the interaction between excitation modality and indicator photophysics has emerged as a critical bottleneck. Voltage indicators based on VSDs generally retain functionality under two-photon excitation, whereas many FRET-opsin indicators exhibit markedly reduced or absent voltage sensitivity under these conditions, even though they perform well with one-photon excitation. This discrepancy raised fundamental questions about how voltage sensing is coupled to excitation modality.

Recent mechanistic studies have clarified why two-photon voltage imaging with FRET-opsin indicators has often fallen short of expectations. Cohen and coworkers showed that voltage sensitivity in FRET-opsin indicators does not arise from the microbial rhodopsin ground state but instead depends on an illumination-driven population of photocycle intermediates in the microbial rhodopsin that are sensitive to membrane potential (Figure 1B).[6] As a result, voltage-dependent fluorescence emerges only when excitation conditions sustain sufficient occupancy of these voltage-sensitive states, making voltage responses strongly dependent on illumination wavelength, intensity, and temporal pattern. This insight explains why two-photon excitation can fail to reproduce one-photon voltage sensitivity even when fluorescence brightness appears comparable, since conventional scanning excitation may excite the fluorophore without effectively driving the microbial rhodopsin through its voltage-sensitive transitions. By adjusting excitation parameters to better populate these intermediates, prior work demonstrated that voltage sensitivity can be recovered under two-photon conditions. At the same time, these findings highlighted a remaining challenge: translating this mechanistic understanding into a general and scalable strategy for high-speed two-photon voltage imaging with fully genetically encoded indicators.

In this issue of *Neuron*, Emiliani and coworkers introduce Jarvis, a FRET-opsin voltage indicator developed in conjunction with a scanless two-photon excitation strategy to enable fast voltage imaging under two-photon conditions.[2] Jarvis incorporates a highly efficient fluorescent protein donor, increasing photon output and improving voltage-dependent fluorescence signals. Using parallel excitation, the authors report the unexpected finding that rhodopsin-based indicators can support robust two-photon voltage

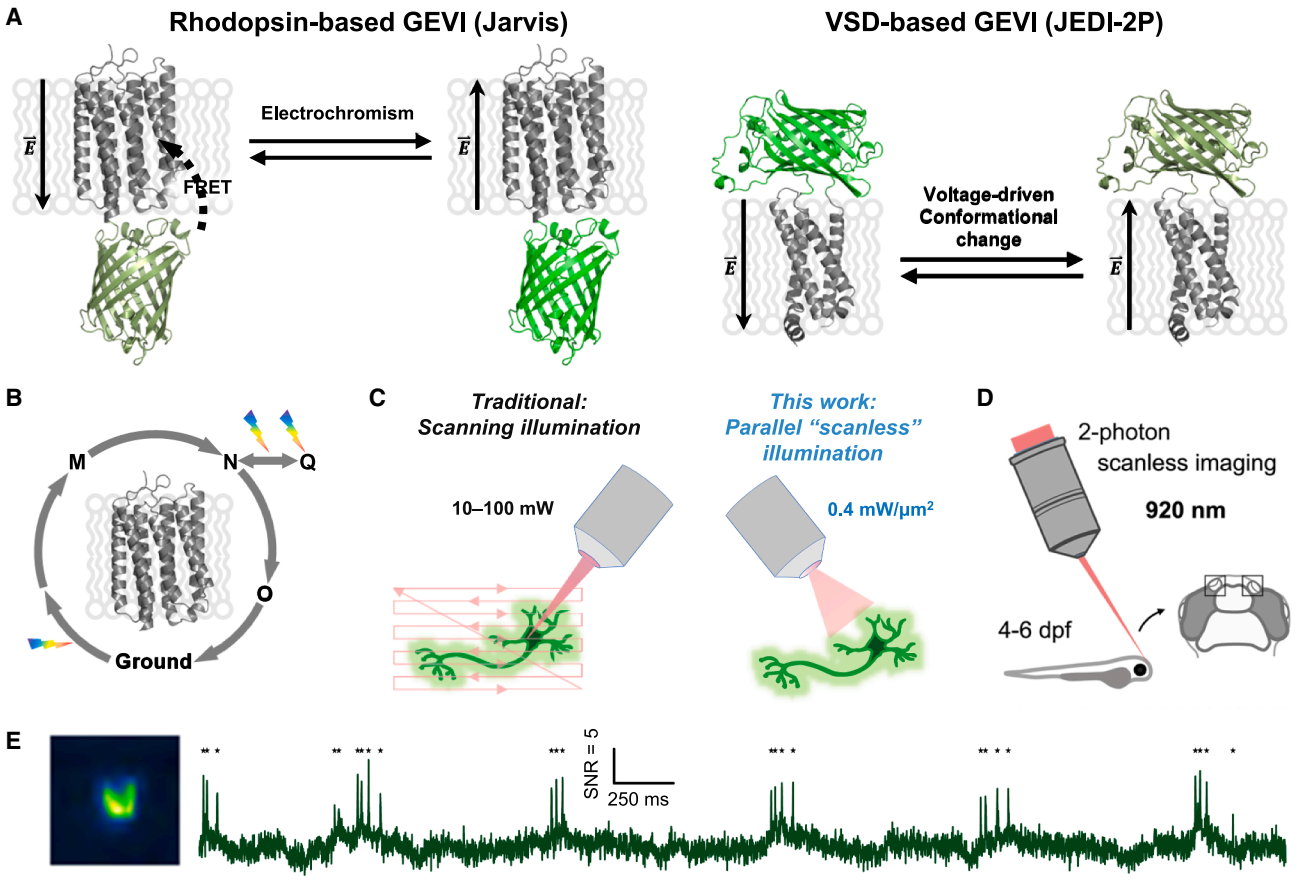

Figure 1. Two-photon imaging with voltage indicators

(A) Scheme of voltage-sensing mechanism of rhodopsin-based GEVIs (i.e., Jarvis) and VSD-based GEVIs (i.e., JEDI-2P).
(B) Scheme of microbial rhodopsin photocycle.
(C) Scheme of traditional scanning two-photon (2P) excitation mode and parallel (scanless) 2P excitation mode.
(D and E) Scheme (D) and optical trace (E) of scanless 2P voltage imaging in larval zebrafish, as demonstrated in the Jarvis paper.[2]

imaging, challenging the prevailing view that such indicators are intrinsically incompatible with this excitation mode. This behavior extends beyond Jarvis itself, as similar responses are observed in other indicators in this class, including pAce[7] and Voltron2.[8] Together, these results identify parallel excitation as a unifying requirement for enabling two-photon voltage imaging with rhodopsin-based indicators across sensing polarities and reporter formats.

The authors directly compared conventional scanning excitation with parallel scanless excitation to determine how illumination mode shapes voltage readout under two-photon conditions (Figure 1C). Although scanning achieves high instantaneous intensity within a diffraction-limited focus, the beam must be rapidly displaced to cover the field of view, resulting in only brief illumination at any given membrane location. By contrast, parallel excitation distributes light across the entire region of interest, providing lower instantaneous intensity but sustained excitation over longer timescales. This difference in temporal pattern has important consequences for rhodopsin-based indicators. Parallel excitation yields brighter voltage-dependent signals and faster response kinetics, consistent with more effective population and maintenance of voltage-sensitive states in the microbial rhodopsin photocycle. These observations support a model in which voltage reporting depends not only on excitation efficiency but also on illumination history, with sustained excitation favoring the light-dependent transitions that enable voltage sensitivity. By resolving this mismatch between excitation modality and indicator photophysics, parallel excitation overcomes a central limitation that has constrained two-photon voltage imaging with these indicators.

Building on this mechanistic foundation, the authors demonstrate that Jarvis combined with scanless two-photon excitation enables voltage imaging in intact neural tissue and *in vivo*. After validation in brain slices, the approach is applied to record spontaneous action potential activity in larval zebrafish, capturing fast voltage dynamics under two-photon conditions in a transparent vertebrate nervous system (Figures 1D and 1E). Crucially, the method also supports voltage imaging in the barrel cortex of awake mice, establishing compatibility with scattering brain tissue and behaviorally relevant contexts. These demonstrations show that scanless two-photon

voltage imaging can resolve rapid electrical signals *in vivo* with temporal precision that has been difficult to achieve with conventional scanning approaches. More broadly, this work positions parallel excitation as a practical strategy for extending voltage imaging beyond superficial preparations and into experimental regimes relevant to circuit neuroscience.

Together with recent mechanistic work, this study establishes that successful two-photon voltage imaging with FRET-opsin indicators depends on illumination-dependent population of voltage-sensitive states in the microbial rhodopsin photocycle rather than on indicator properties defined under one-photon excitation alone. From this perspective, voltage sensitivity emerges from the interaction between indicator photophysics and the temporal pattern of excitation, including illumination history and duty cycle. This insight highlights the need to evaluate and optimize voltage indicators directly under two-photon conditions, as irradiance can shape signal amplitude, response kinetics, and signal-to-noise ratio simultaneously. At the same time, the work places new emphasis on the development and broader deployment of scanless two-photon instrumentation, which is integral to implementing photophysics-informed excitation strategies *in vivo*. By conserving photon budget while maintaining voltage-sensitive state populations, parallel excitation offers a practical route toward scalable *in vivo* voltage imaging and underscores the importance of continued codesign of indicators, excitation strategies, and instrumentation.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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