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Lighting up action potentials with fast and bright voltage sensors

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Three groundbreaking studies have created a new generation of genetically encoded voltage indicators, empowering us to tackle a host of questions on our path toward understanding the brain.

Action potentials are depolarization events in neuronal membranes that enable rapid communication between brain cells. Fast computing neurons communicate with both neighboring and distant synaptic partners at high speeds (1–100 m s^{-1}). To understand the brain's complex network of neurons, it is vital to simultaneously record action potentials from a large population of neurons with cell-type specificity (Fig. 1a). Three recent papers in *Nature Methods*[1–3] introduce innovative methods to visualize and measure neuronal communication, presenting neuronal firing as a visual display of illuminated cells during action potential propagation.

Traditionally, electrophysiological techniques using electrodes have been used to measure action potentials, revealing the kinetics of fast signal spiking (1–2 ms duration). However, these methods are invasive and have limited throughput. More recent technologies, such as neuropixels[4], can increase the scale of recordings with parallel microelectrodes but still require invasive brain implants and lack cell-type specificity.

Protein-based sensors, such as genetically encoded voltage indicators (GEVIs), are encoded by DNA and can be targeted to specific cell types. Although the first GEVIs were developed in 1997 (ref. 5), it wasn't until the early 2010s that three major types of GEVIs were established[6], paving the way for non-invasive imaging of large neuronal populations with single-cell resolution. Each type of voltage sensor is distinguished by the molecular components responsible for detecting membrane depolarization and transmitting the signal, and has strengths and weaknesses. The first type employs a voltage-sensitive domain from a phosphatase connected to a fluorescent protein, resulting in bright sensors compatible with two-photon imaging but having relatively slow kinetics. The second type utilizes modified microbial rhodopsins, which naturally respond to voltage changes by altering their fluorescence properties, although they have weak emission and limited compatibility with two-photon imaging. This limitation can be mitigated by coupling with a secondary fluorophore. The third type consists of hybrid chemogenetic sensors, which use a protein tag for localization and an exogenously introduced voltage-sensitive dye. However, the delivery of synthetic dyes and their recycling lifetimes present challenges for long-term, continuous imaging.

Despite the successes in voltage imaging, the brief duration of action potentials make them a challenge to capture, imposing considerable demands on sensors. Fast kinetics, exceptional brightness and precise membrane localization are crucial factors to ensure a high signal-to-noise ratio during the short action potential period, as well as accurate and reliable detection[7]. By engineering sensors to meet these demands, a highly sought-after feature of voltage sensors — the ability to 'switch on' during depolarization — can be achieved. Most current sensors are negative-response sensors that 'switch off' upon depolarization, which imposes limitations due to the decreased signal accuracy in high-background environments. Protein engineering is challenging, as these membrane proteins require a mammalian expression platform, and there is limited knowledge of their structure–function relationship. Screening often involves exploring vast libraries of tens or hundreds of thousands of variants to cover a broad range of amino acid sequences. Additionally, from a signal collection standpoint, enhancements in imaging systems, such as kilohertz scanning of extensive fields of view, are needed to accommodate the high frame rates required for capturing events with 1–2 ms durations.

Substantial effort has thus been made to further improve sensors' performance for in vivo imaging. Platisa and colleagues[1] addressed the issue of photon scarcity by developing a platform for low photon flux imaging and designing positive-response sensors. Through targeted protein engineering, they evolved SpikeyGi and SpikeyGi2 (derived from ASAP3), both of which exhibit increased fluorescence in response to voltage depolarization. They also constructed a two-photon microscope that combines spatial and temporal multiplexing with rapid imaging over a large field of view. In addition, they created a deep convolutional neural network for signal denoising to extract data from recordings limited by shot noise. Employing low-power illumination, they captured up to 60 min of continuous images of spontaneous firing with minimal photobleaching. Their denoising algorithm enabled the extraction of individual firing events from photon-limited traces, achieving a high signal-to-noise ratio (Fig. 1b).

Evans and colleagues[2] built upon their previous work by re-engineering an existing GEVI, ASAP2, into two positive-response sensor variations: ASAP4b and ASAP4e. These members of the ASAP family achieve a two- and fourfold maximum change in fluorescence upon depolarization, respectively, a substantial improvement compared to the ~51% modulation of the best sensor in the same family (ASAP3). The fast kinetic response of these sensors is demonstrated by their ability to detect action potential spikes 1 ms long at 1,000 Hz, making them suitable for resolving spontaneous firing of neurons in vivo with high fidelity.

Another advantage of positive-response sensors is their low brightness baseline, which results in lower photobleaching rates. Evans et al. showed that they can measure the signal from ASAP4 for tens of minutes with negligible bleaching under one- and two-photon illumination. They also implemented a spike detection framework to improve spike recognition on the basis of the nature of the noise (Gaussian-like or Poisson-like). In a sophisticated in vivo demonstration, the ASAP4 sensors were co-expressed with the red calcium indicator jRGECO1a, allowing enriched information extraction about firing neurons.

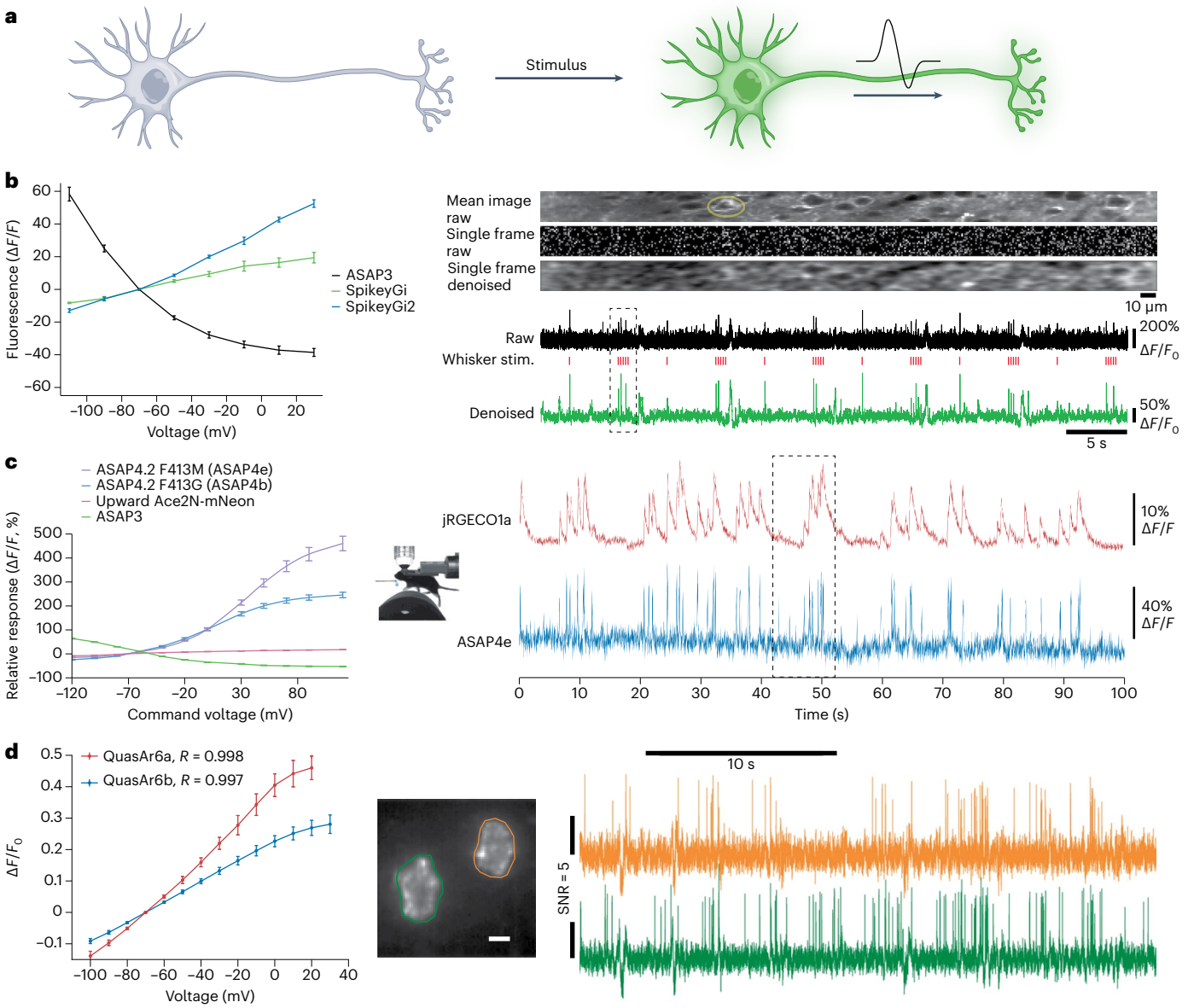

Fig. 1 | Fast voltage sensors to highlight action potentials in neurons, showing fluorescence response to voltage and an example of spikes recording in vivo. a, Genetically encoded voltage indicators light up the neuronal membrane during action potentials. **b**, Left: positive response of the SpikeyGi and SpikeyGi2 sensors as a function of depolarization potential. Right: imaging of layer 2/3 neurons in vivo expressing SpikeyGi2 using ultrafast kilohertz image acquisition developed by the authors and showing the power of a neural network-based deconvolution algorithm in denoising traces to identify signals. Adapted from ref. 1, Springer Nature. **c**, Left: response to voltage changes of the ASAP4 sensor family compared to existing GEVIs, showing the increased dynamic range and sensitivity of the new sensors. Right: in vivo imaging of ASAP4e, co-expressed in layer 2 neurons with a calcium indicator. The data are raw traces collected during a reward experiment, showing the high signal-to-noise ratio (SNR) of the sensor and displaying the advanced temporal resolution achievable with this GEVI, as compared to calcium detection. Adapted from ref. 2, Springer Nature. **d**, Left: response range and high linearity of the QuasAr6 sensor family, with positive increase in fluorescence upon depolarization. Right: in vivo monitoring of spontaneous dynamics of a pair of parvalbumin neuron imaged at 2 kHz; fast spike trains are visible and single action potentials are clearly distinguishable. Scale bar, 10 µm (centre panel). Adapted from ref. 3, Springer Nature.

The most remarkable feature of these ASAP4 sensors is their ability to report single action potentials in vivo with exceptional clarity, as seen in the raw data (Fig. 1c). This clarity means that measuring the speed of neuronal firing does not require complex data processing tools; instead, software widely used in the brain imaging community can successfully analyze the data.

Finally, Tian and colleagues[3] developed two rhodopsin-based voltage sensors derived from Archon1, a well-established template

for other GEVIs[8]. Rhodopsin-based GEVIs possess naturally fast on/off kinetics but often suffer from low brightness, leading to a poor signal-to-noise ratio for in vivo measurements.

To address the low brightness, the authors combined directed evolution of the sensor in mammalian cells with their Photopick platform[9], using a pooled approach. The authors imaged a large field of view containing thousands of cells expressing unique variants. After stimulating the cells, they labeled those with the most interesting responses using a fluorescent protein. After isolating the marked cells, they identified the mutations responsible for the improved phenotype. The authors focused on selecting an average improved phenotype rather than isolating individual outliers, aiming to introduce mutations that would result in low cell-to-cell variability and more reliable readouts. Consequently, they developed QuasAr6a and QuasAr6b, both of which are brighter than their parent template. QuasAr6b demonstrates exceptional kinetics, with sub-millisecond on and off times.

As a proof of principle, Tian and colleagues capitalized on their improved sensors to image hippocampal parvalbumin neurons, which display fast and narrow spikes, typically posing challenges for optical imaging and patch clamping due to their difficult targeting. The authors recorded correlated voltage dynamics between pairs of hippocampal parvalbumin neurons for both subthreshold fluctuations and spikelets (spatially confined action potentials) and confirmed that QuasAr6 is suitable for recording fast voltage signaling (Fig. 1d).

These groundbreaking papers showcase how innovative methods in screening platform, microscopy and data analysis can rapidly advance the development and applications of more accurate sensors to record action potentials in living animals. We eagerly anticipate this technique becoming as widespread and indispensable for neuroscience research as calcium imaging. The creation of new molecular tools also drives the development of optical setups for faster and higher throughput imaging, as demonstrated by Platisa et al., pushing the boundaries of what can be achieved in an 'all-optical electrophysiology' platform. While constructing a microscope may present challenges and limit the applicability of some sensors, we hope that this progress inspires further development of open-source, self-contained microscopy solutions that can be easily deployed across laboratories. This will pave the way for 'needleless' recording of firing neurons to become a routine measurement. Let the visual sparks illuminate the path to discovering intricate connections in the mesmerizing depths of brain circuitry.

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Competing Interests

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