Two-photon *in vivo* imaging of neuronal membrane potential with VoltageFluor

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Summary

Optical imaging offers unique opportunities for obtaining minimally invasive measurements of neuronal activity in the living brain. However, in vivo imaging of neuronal membrane potential remains a challenge due to poor performance of the existing voltage probes under multiphoton excitation. Here, we demonstrate 2-photon membrane potential imaging in vivo and in vitro using a voltage-dependent photo-induced electron transfer (PeT) sensor VF2.1(OMe).H [1,2].

Methods

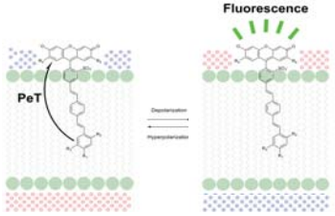


Fig. 1. Voltage sensing mechanism: Depolarized membrane potentials (negative inside the cell) promote PeT and *quench fluorescence*. Hyperpolarization decreases PeT and *increases fluorescence*. Thus, the quantum yield of VF dyes are related to the local membrane potential [1,2].

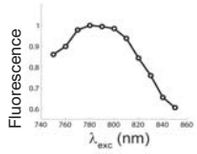
Voltage indicator VoltageFluor (VF2.1(OMe).H) (Fig. 1) was first dissolved in 4 µl of 20% pluronic in DMSO (F-127, Invitrogen); 80 µl of artificial CSF (ACSF) containing 142 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM Hepes, 3.1 mM CaCl2, 1.3 mM MgCl2, pH 7.4 were added to the solution to yield a final concentration of 0.5 mM VF. The VF solution was pressure-microinjected into the cortical tissue (either a cortical slice or *in vivo* cortex).

Neocortical slices were prepared according to standard procedures [3]. We whole-cell patched cell bodies of layer V PCs within the labeled tissue volume 50-80 µm below the surface of the slice. Alexa 594 Fluor (20 µM) was added to the intracellular solution to determine the plasma membrane boundary. Spikes were driven by 5-ms square current pulses with amplitude adjusted to generate an AP.

***In vivo* animal procedures:** Mice were imaged under α-chloralose anesthesia. ~2x2 mm cranial window had a gap on the lateral side to allow insertion of the pipette for VF loading and tungsten electrode for LFP recordings.

Sensory stimulation was delivered to the forepaw contralateral to cortical exposure through a pair of thin needles inserted under the skin using 100-µs, ~1-mA electrical pulses.

Two-photon imaging: Images were obtained using an Ultima 2-photon laser scanning microscopy system. VF was excited at 800 nm.

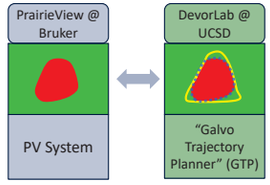


To generate ratio images (Fig. 2), we imaged ~50x50 µm field of view (FOV) containing the cell body in a frame scan mode at ~20 frames per second.

For detection of single spikes, we implemented line scans at ~0.5 ms per line. Each line contained ~ 50 pixels using 10 µs dwell time per pixel.

Procedure to define line scan trajectory:

- Start external "Galvo Trajectory Planner" (GTP)
- Grab current PV screen image into GTP
- User draws desired trajectory on the image
- GTP calculates optimal galvo trajectories using calibration data
- GTP generates x-y coordinates as PV line scan trajectory file
- User imports the x-y list to PV



[1] Miller et al., PNAS 2012 Feb 7;109(6):21149
[2] Woodford et al., J Am Chem Soc. 2015 Feb 11;137(5):181724
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2-photon imaging in brain slices

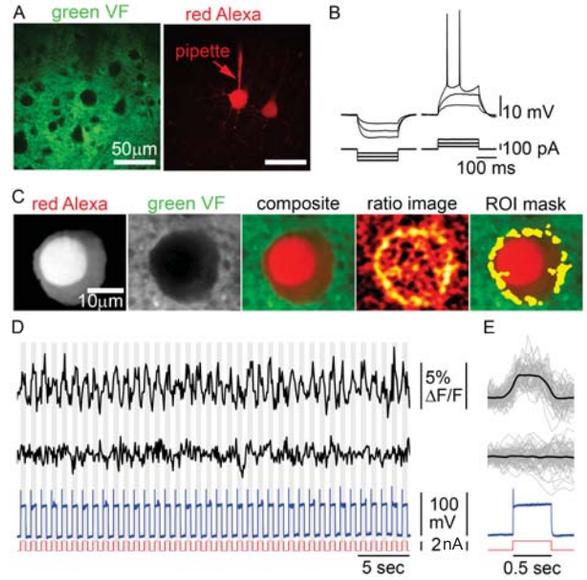

Fig. 2. VF signal outlines the plasma membrane and follows membrane voltage. A. Left: VF-loaded slice. Right: Layer 2/3 PC with a patch electrode filled with Alexa 594. **B.** Whole-cell current-clamp. **C.** Example FOV, ratio image of VF signal, and the corresponding ROI mask. **D.** VF signal time-course extracted from the ROI (upper black trace) and outside (lower black trace). Simultaneous whole-cell patch recording is shown below. **E.** Respective trial-averaged time-courses (n ≈ 40 trials).

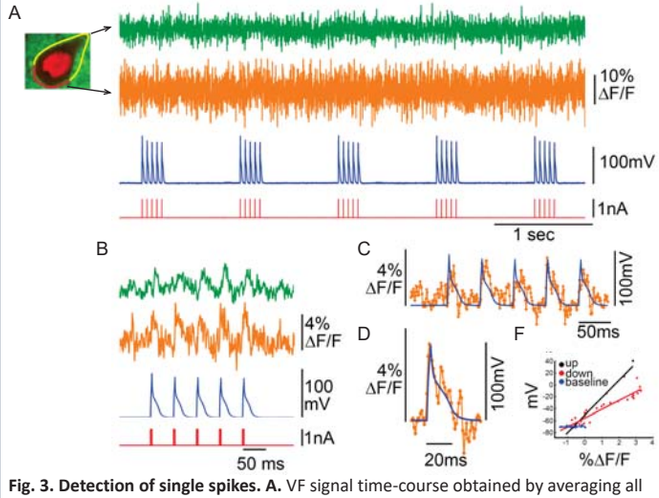

Fig. 3. Detection of single spikes. A. VF signal time-course obtained by averaging all pixels along the trajectory (top) and selected segment (bottom). Simultaneous whole-cell patch recording is shown below. **B.** Trial-averaged time-courses (n ≈ 40 trials). **C.** Overlaid trial averaged VF signal and membrane potential from (B). **D.** Grand average for a single spike. **E.** Fluorescence as a function of the membrane voltage. Values during the baseline, voltage sweep up, and voltage sweep down are color-coded.

2-photon imaging *in vivo*

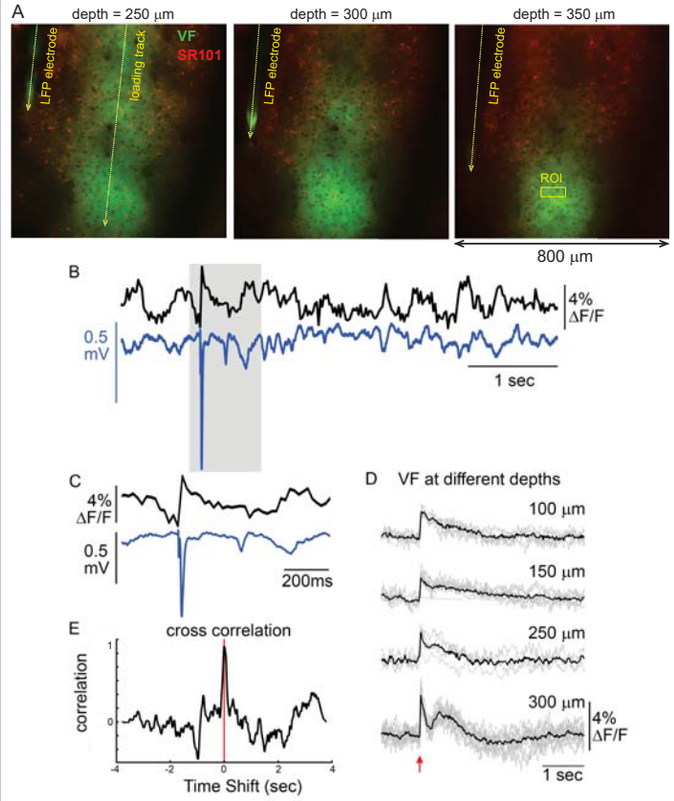

Fig.4. VF signal *in vivo* as the local measurement of neuropil voltage. A. *In vivo* loading. **B.** VF signals extracted from the ROI shown in (A) (black) and simultaneously recorded LFP (blue). **C.** Zoomed in view of the segment indicated by the gray rectangle in (B). **D.** VF signal time-courses at different depths. **E.** Cross-correlation between VF fluorescence and LFP during spontaneous activity.

Conclusions and Outlook

1. VF2.1(OMe).H can be used to obtain local neuropil voltage *in vivo*
2. Negativity in the LFP accompanied by depolarization in layer 2/3 is compatible with a local current sink and excitatory synaptic inputs
3. Combination of 2-photon VF imaging with laminar LFP recordings would allow underpinning of current sinks and sources

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