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High-resolution simultaneous voltage and Ca\(^{2+}\) imaging

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**Revised version**
Combining voltage and Ca\(^{2+}\) imaging allows the correlation of electrical and chemical activity at sub-cellular level. Here we describe a novel apparatus designed to obtain simultaneous voltage and Ca\(^{2+}\) measurements with single-trial resolution from sites as small as a few microns. These measurements can be obtained with negligible optical cross-talk between the two signals and negligible photo-damage of the preparation. The capability of the technique was assessed recording either from individual neurons in brain slices or from networks of cultured neurons. The present achievements open the gate to many novel physiological investigations requiring simultaneous measurement of voltage and Ca\(^{2+}\) signals.

**List of abbreviations:**

CF, climbing fibre; EPSP, excitatory synaptic potential; PF, parallel fibre; PN, cerebellar Purkinje neuron; 
S/N, signal-to-noise ratio; \(\Delta V_m\), membrane potential changes.

**Introduction**

The spatial and temporal correlation of membrane potential changes (\(\Delta V_m\)) and Ca\(^{2+}\) signals is critical to understanding many physiological processes. Simultaneous \(\Delta V_m\) and Ca\(^{2+}\) optical measurements were initially achieved using a combination of an absorbance voltage sensitive dye and of a fluorescent Ca\(^{2+}\) indicator (Sabatini & Regehr, 1996), or of fluorescent voltage and Ca\(^{2+}\) sensitive dyes excited by the same wavelength (Sinha *et al.* 1995; Bullen & Saggau, 1998). Whereas absorbance measurements lack single-cell resolution, several limitations affect fluorescence measurements of \(\Delta V_m\) and Ca\(^{2+}\) signals excited at the same wavelength. In particular, the optical cross-talk of the two dyes contaminates both signals while using the same excitation intensity and exposure time is not ideal for both dyes. These limitations can be minimised by combining a styryl voltage sensitive dye such as di-2-ANEPEQ (JPW1114) with Fura Ca\(^{2+}\) indicators, separating both excitation and emission (Canepari *et al.* 2008). Such a combination was used to investigate \(\Delta V_m\) and Ca\(^{2+}\) signals in different preparations (Canepari *et al.* 2007; Canepari & Vogt, 2008; Milojkovic *et al.* 2008). This approach, however, utilised sequential repetitive recordings of \(\Delta V_m\) and Ca\(^{2+}\) signals. Sequential measurements (see also Berger *et al.* 2007) give valid results if identical responses are evoked in repetitive measurements. Many physiological processes, however, are stochastic, showing large trial-to-trial variability. In these cases, the study of the time variability contains important biological information and simultaneous measurement of \(\Delta V_m\) and Ca\(^{2+}\) signals with single-trial resolution is required.
For simultaneous voltage and Ca\(^{2+}\) imaging using commercially available styryl voltage sensitive dyes and Fura Ca\(^{2+}\) indicators, a system must be designed to illuminate the preparation at two wavelengths and record \(\Delta V_m\) and Ca\(^{2+}\) signals separately. Here we describe a system developed for this application. We used different protocols to characterise the signal-to-noise ratio (S/N) and the spatial and temporal resolution. We demonstrate that \(\Delta V_m\) and Ca\(^{2+}\) signals associated with excitation can be resolved in a single trial from sites of a few microns.

**Methods**

**Preparations, solutions, electrophysiology and analysis**

Experiments, approved by Basel cantonal authorities, were performed in 250µm thick cerebellar sagittal slices or hippocampal transversal slices from 27-35 days old mice (C57BL/6) prepared as previously described (Canepari & Vogt, 2008; Canepari et al. 2010). Embryonic (E18) hippocampal cultures were prepared by Dr. Alexander Kriz as described in Reiterer et al. (2008) and used after 10-14 days *in vitro*. The extracellular solution contained (mM): 125 NaCl, 26 NaHCO\(_3\), 20 glucose, 3 KCl, 1 NaH\(_2\)PO\(_4\), 2 CaCl\(_2\) and 1 MgCl\(_2\), bubbled with 95% O\(_2\) and 5% CO\(_2\). The intracellular solution contained (mM): 125 KMeSO\(_4\), 5 KCl, 8 MgSO\(_4\), 5 Na\(_2\)-ATP, 0.3 Tris-GTP, 12 Tris-Phosphocreatine, 20 HEPES, adjusted to pH 7.35 with KOH. Patch-clamp recordings were made using a Multiclamp amplifier 700A (Molecular Devices, Sunnyvale, CA) and stimulation was carried out with pipettes filled with extracellular solution. Individual neurons in slices were loaded with JPW1114 (0.2-0.5mg/mL) and with either 1 mM Fura-FF (Purkinje neurons) or with 0.5mM BisFura-2 (pyramidal neurons). Intracellular staining was accomplished as previously described (Canepari et al. 2008). Cultures were stained with di-8-ANEPPS (0.01 mg/mL) and Fura2-AM (5 µM) for 30 minutes and recordings were done after wash. Temperature was 32°-34° in slice experiments and 22°-24° in culture experiments. Recordings were analysed using Matlab (The MathWorks Inc., Natick, MA). Non-calibrated optical signals were reported as fractional changes in fluorescence (\(\Delta F/F\)). Anatomical reconstruction of neurons was made from two-photon images as described in Canepari et al. (2010).

**Apparatus design and data collection**

The system in Fig. 1A, used and described in Canepari et al. (2008), was based on a single light source (Xenon Lamp) and a single camera (NeuroCCD-SM, RedShirtImaging LLC, Decatur, GA). Voltage and
Ca\(^{2+}\) imaging was carried out by manually switching between two filter cubes in the microscope. To achieve simultaneous voltage and Ca\(^{2+}\) imaging we modified the system as shown in Fig. 1B. The epifluorescence port of an Olympus BX51 microscope was used for Ca\(^{2+}\) fluorescence excitation with a 385 nm OptoLED (CAIRN Research Ltd., Faversham, UK), filtered at 387±6 nm and directed to a Nikon 60X/1.0 NA objective using a dichroic mirror FF506 (Semrock, Rochester, NY; spectrum shown in Fig. 1B). To excite voltage fluorescence, a custom-made unit was designed permitting whole-field illumination of the preparation from above the objective using a 100 mW 543 nm solid state laser (model MLL543; CNI, Changchun, China). To reflect 543 nm and transmit voltage and Ca\(^{2+}\) fluorescence we used a dual-band dichroic mirror FF493_574 (Semrock; spectrum shown in Fig. 1B). The image of the preparation was demagnified by a 0.5X projection lense in both voltage and Ca\(^{2+}\) channels and separated using another custom-made unit and a 565 nm long-pass dichroic mirror (565DCXR, Chroma, Bellows Falls, VT). Voltage fluorescence was long-pass filtered at 610 nm (em1 in Fig. 1B), the image was demagnified by 0.38X and acquired by a NeuroCCD-SM camera. Ca\(^{2+}\) fluorescence was filtered at 510±42 nm (em2 in Fig. 1B), the image was demagnified by 0.38X and acquired by a back-illuminated Ixon+ model 860 EMCCD camera (Andor, Belfast, UK), controlled by the Redshirt system A/D board. The image on the 80X80 pixel chip of the NeuroCCD camera could be matched and aligned with the image projected to a sub-region of 78X78 pixels of the Ixon+ camera, corresponding to ~160X160 µm area in the object plane as shown in Fig. 1B. Measurements reported in this article were taken at 2000 Hz with the NeuroCCD (voltage indicator fluorescence) and 751.88 Hz (slices) or 200 Hz (cultures) with the Ixon+ (Ca\(^{2+}\) indicator fluorescence). The time information of simultaneous voltage and Ca\(^{2+}\) imaging measurements is limited by the sampling theorem as discussed in the Supplementary Information.

S/N and optical cross-talk

Fig. 1C shows single-trial sequential recordings from a region of ~12.5X12.5 µm obtained with the system in Fig. 1A. The signals are associated with a climbing fibre (CF) excitatory postsynaptic potential (EPSP) from a cerebellar Purkinje neuron (PN). In Canepari & Vogt (2008) CF-EPSP signals could be resolved from regions of that size by averaging 4 trials (bottom traces in Fig. 1C). Fig. 1D shows single-trial simultaneous recordings from a region of ~12X12 µm obtained with the system in Fig. 1B. The improvement in voltage sensitivity using a laser at the edge of the absorption spectrum (see also Holthoff et al. 2010 and Canepari et al. 2010) and the higher UV intensity of the OptoLED resulted in the S/N increase necessary for single-trial resolution. Whereas negligible cross-talk is observed by the Ixon+ camera, the
simultaneous UV illumination generated an increase in the recorded light by the NeuroCCD camera. This spurious fluorescence, originating both from the tail UV excitation of the voltage sensitive dye and from the tail red emission of the Fura indicator, could vary between 3% and 10% at different sites of the preparation (Fig. 1E, left traces) using the optimal intensities of UV and green excitation. Nevertheless, the $\Delta V_m$ signal was the same whether or not measured with concomitant UV illumination (Fig. 1E, right traces), indicating that optical cross-talk was negligible in these measurements.

Results

We assessed the spatial resolution of $\Delta V_m$ and Ca$^{2+}$ signals in individual PNs (Canepari & Vogt, 2008). In this preparation, $\Delta V_m$ signals can be calibrated and, using 1 mM of the low-affinity indicator Fura-FF, Ca$^{2+}$ signals can be related to intracellular free Ca$^{2+}$ concentration changes ($\Delta [\text{Ca}^{2+}]_i$) (Canepari et al. 2008). Thus, a CF-EPSP producing a depolarization of similar size over the whole dendrite was used to assess and compare the sensitivity from different sites. In contrast, a parallel fibre (PF) EPSPs train, exciting only a small area, was used to assess to what extent correlated and localized $\Delta V_m$ and Ca$^{2+}$ signals could be resolved. Fig. 2A-C shows an example of CF-EPSP single-trial recording. The S/N of both signals was variable. Fig. 2B shows the $\Delta V_m$ and $\Delta [\text{Ca}^{2+}]_i$ signals from three regions of 16X16 pixels (~32X32 µm) in different parts of the dendrite. Signals could be resolved with excellent S/N from all the three regions and from regions of the same size in all the cells tested (N=12). Signals with higher spatial resolution could also be obtained from several areas of each neuron. Fig. 2C shows three sub-regions, from region 3 in panel B, covered by 1 pixel (~2X2 µm), 3X3 pixels (~6X6 µm) and 5X5 pixels (~10X10 µm). Both $\Delta V_m$ and $\Delta [\text{Ca}^{2+}]_i$ signals could be detected from the single pixel and the S/N increased in the two larger regions. In summary, in all cells tested, $\Delta V_m$ and $\Delta [\text{Ca}^{2+}]_i$ signals could be resolved from regions <20 µm$^2$.

We next performed a series of experiments (N=4 cells) in which dendritic signals were elicited by trains of PF-EPSPs. In the example of Fig. 2D-F, the $\Delta V_m$ and $\Delta [\text{Ca}^{2+}]_i$ signals associated with one CF-EPSP and a train of 5 PF-EPSPs at 100 Hz were compared in two 8X8 pixels regions. $\Delta V_m$ and $\Delta [\text{Ca}^{2+}]_i$ signals associated with large depolarization could be observed in both regions for the CF-EPSP but only in region 1, near the PF stimulating electrode, for the PF-EPSP train. Because the $\Delta [\text{Ca}^{2+}]_i$ originates from Ca$^{2+}$ influx during the spikes (Canepari & Vogt, 2008), it is expected to observe a qualitative spatial correlation between the peak $\Delta V_m$ and $\Delta [\text{Ca}^{2+}]_i$ signals. Thus, in Fig. 2G, we compared the $\Delta V_m$ and $\Delta [\text{Ca}^{2+}]_i$ signals in the 3X3 pixels maximal region and in the progressively more distal 5X3 pixels, 7X3 pixels and 9X3 pixels
regions. Both the $\Delta V_m$ and $\Delta [Ca^{2+}]_i$ decreased with distance from the 3X3 pixels maximal region, a result confirmed in the 4 cells (Fig. 2H). This result indicates that single-trial measurements were able to identify sites with correlated $\Delta V_m$ and $\Delta [Ca^{2+}]_i$ signals with a spatial resolution of ~6 µm.

Fig. 3A-C illustrates the more common scenario in which $\Delta V_m$ and $\Delta [Ca^{2+}]_i$ signals vary in time, as recorded from individual CA1 hippocampal pyramidal neurons in brain slices. The signals were evoked by applying tetanic stimulation (10 pulsed at 100 Hz) to the CA3 region (Fig. 3A). Under control conditions, shown in Fig. 3A-B, stimulation elicited three action potentials that back-propagated in the apical dendrite with random timing. We then applied 50 µM of the GABA$_A$ receptor antagonist picrotoxin to block synaptic inhibition and monitored the evoked activity every three minutes. The number and frequency of action potentials increased with time (see also SupplementalMovie1). The capability of the technique to resolve $\Delta V_m$ and $\Delta [Ca^{2+}]_i$ signals in single trials was critical in this experiment.

We finally tested whether our system can be used in experiments from cell cultures labelled by bulk application of the dyes. Fig. 3D shows three images from a culture dish obtained by di-8-ANEPPS fluorescence (vf), Fura-2 fluorescence (cf) and transmitted light (tr). Individual cells were clearly visible. Single-trial recordings shown in Fig. 3E show that $\Delta V_m$ and $\Delta [Ca^{2+}]_i$ signals associated with one or four evoked action potentials could be measured exclusively from the stimulated cell 1 (see also SupplementalMovie2).

**Discussion**

In this report we describe how to achieve simultaneous voltage and Ca$^{2+}$ imaging with S/N permitting single-trial resolution. Signals of ~1% change of fluorescence could be discriminated from regions of a few microns. In voltage imaging, 1% signal typically corresponds to $\Delta V_m$~10-20mV in individually stained cells or in cultured cells after bulk staining. Thus, activity could be monitored from small sub-cellular compartments. For Ca$^{2+}$ imaging, the size of the optical signal corresponding to a given Ca$^{2+}$ signal depends on the affinity and concentration of the dye. However, we showed that Ca$^{2+}$ signals associated with individual action potentials could be resolved both with low and high affinity dyes. Two concerns that limit the applicability of the technique are photo-toxicity of the voltage sensitive dye and optical cross-talk. Full illumination with a 100 mW 543 nm laser is equivalent, in terms of dye absorption, to ~25% illumination with a 300 mW 532 nm laser. In this study, using full-light illumination at 543 nm, we did not detect any photo-damage for at least 20 sequential measurements of 100 ms duration, separated by 30 s.
This result is consistent with our previous tests at 532 nm (Canepari et al. 2010). In voltage fluorescence measurement, UV illumination generates a fluorescence increase that depends on the concentrations of the two dyes (Fig. 1E). This spurious fluorescence, however, didn’t affect the ∆V_m signal.

The present results open the gate to novel investigations requiring simultaneous measurement of ∆V_m and Ca^{2+} signals, particularly in neuro-physiology and cardio-physiology. The apparatus is based on commercially available equipment that can be adapted to conventional microscopes.

References


**Author contribution**

Design of the system K.V., J.G., M.C. Design of the experiments: K.V., M.C. Performing the experiments S.G., M.C. Writing and revising the article M.C. - All authors approved the final version.

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**Figure Legend**

**Figure 1. Imaging apparatus and sensitivity**

A, Schematic drawing of the imaging apparatus used in Canepari & Vogt (2008); combined voltage and Ca\(^{2+}\) imaging obtained sequentially by switching between two filter cubes. B, Schematic drawing of the apparatus for simultaneous voltage and Ca\(^{2+}\) imaging: 385 nm OptoLED (CAIRN) illumination via the epifluorescence port of the microscope reflected by a dichroic mirror FF506 (Semrock, transmission curve shown on the right); 543 nm laser illumination via the top of the microscope is reflected by a dual-band dichroic mirror FF493_574 (Semrock, transmission curve shown on the right); the image in the V\(_m\) and Ca\(^{2+}\) channels are demagnified by 0.5X projection lens, separated by a 565 nm dichroic mirror (dic, 565DCXR, Chroma) and filtered by a 610 nm long-pass filter (em1, RG610, Schott) and a 510±42 nm band-pass filter (em2, FF01-510/84, Semrock); the two images were further demagnified by 0.38X projection lens before being acquired by a NeuroCCD camera (Redshirt) and Ixon+ EMCCD camera (Andor); two aligned images of a micrometer scale are shown. C, V\(_m\) (red traces) and Ca\(^{2+}\) (blue traces) signals associated with a CF-EPSP from the region (~12.5X12.5 µm) indicated below obtained with the apparatus in A: single trials (top) and averages (bottom). D, simultaneous V\(_m\) and Ca\(^{2+}\) signals associated with a CF-EPSP from the region (~12X12 µm) indicated below obtained with the apparatus in B: single trials (top) and averages (bottom). Notice the S/N increase permitting single-trial resolution. E, V\(_m\) signals associated with a CF-EPSP from the 3 regions indicated on the left with (red traces) and without (gray traces) concomitant UV illumination; superimposed traces (right) show no signal change with UV illumination.

**Figure 2. Simultaneous V\(_m\) and Ca\(^{2+}\) measurements from cerebellar Purkinje neurons**

A, reconstruction of a PN; the dendritic area in recording position is outlined. B, left: fluorescence image with 3 selected regions of interest (16X16 pixels; ~32X32 µm) outlined; right: ΔV\(_m\) (red traces) and Δ[Ca\(^{2+}\)]\(_i\) (blue traces) signals associated with a CF-EPSP (single trial) from the three regions of interest; somatic electrical recording shown below. C, left: region of interest 3 from panel B, 3 subregions of 1 (2 µm), 3X3 (6 µm) and 5X5 pixels (10 µm); right ΔV\(_m\) and Δ[Ca\(^{2+}\)]\(_i\) signals from the three subregions. D, reconstruction of another PN with the dendritic area in recording position outlined. E, fluorescence image with three selected regions of interest (8X8 pixels; ~16X16 µm) outlined. F, ΔV\(_m\) (red traces) and Δ[Ca\(^{2+}\)]\(_i\) (blue traces) signals associated with a CF-EPSP (left) or with a train of 5 PF-EPSPs at 100 Hz (right) from
the three regions of interest; somatic electrical recordings shown below. G, left: same cell as in D-F, the 3X3 pixels region of maximal $\Delta V_m$ and $\Delta [Ca^{2+}]_i$ (d0) and the progressively more distal regions of 5X3 pixels (d1) 7X3 pixels (d2) and 9X3 pixels (d3); right, $\Delta V_m$ and $\Delta [Ca^{2+}]_i$ signals associated with the PF-EPSPs from regions d0-d3. H, $\Delta V_m$ and $\Delta [Ca^{2+}]_i$ peak amplitudes as a function of location associated with a train of 5 PF-EPSPs from 4 cells: d0 is the 3X3 pixels region of maximum $\Delta V_m$ and $\Delta [Ca^{2+}]_i$ amplitudes. In all cells both signals decay with distance.

**Figure 3. Simultaneous $V_m$ and $Ca^{2+}$ measurements from hippocampal neurons**

A, left: schematic drawing of the hippocampus with stimulating electrode in the CA3 region and the recording site in the CA1 region of a pyramidal neuron; right- reconstruction of a neuron (top) and the recording area outlines (bottom); B, $\Delta V_m$ (red traces) and $\Delta [Ca^{2+}]_i$ (blue traces) single-trial signals from the region of interest in A associated with randomly occurring action potentials evoked by CA3 tetanic stimulation (10 pulses at 100 Hz) in control conditions; recordings were done every minute. C, same as B after addition of 50 µM picrotoxin; recordings were done every 3 minutes; animation of this experiment in SupplementalMovie1. D, images from hippocampal cultured cells stained with di-8-ANEPPS and Fura-2-AM; from left to right: voltage sensitive dye fluorescence image (vf), $Ca^{2+}$ indicator fluorescence image (cf) and image under transmitted light; regions of interest (1-3) from three identified cells are indicated. E, $\Delta V_m$ (red traces) and $\Delta [Ca^{2+}]_i$ (blue traces) single-trial recordings from the regions of interest in D associated with 1 (left) or 4 (right) action potentials evoked in cell 1. Signals were localised; animation in SupplementalMovie2.
FIGURE 1
FIGURE 3

A

CA1

CA3

Stim. 10 pulses @ 100 Hz

B

control

2%

4%

50 ms

C

After addition of 50 μM picrotoxin

3 mins

6 mins

9 mins

2%

4%

50 ms

D

vt

cf

tr

50 μm

E

1

4% 20%

50 ms

2

3

FIGURE 3