Imaging with organic indicators and high-speed charge-coupled device cameras in neurons: some applications where these classic techniques have advantages

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Abstract. Dynamic calcium and voltage imaging is a major tool in modern cellular neuroscience. Since the beginning of their use over 40 years ago, there have been major improvements in indicators, microscopes, imaging systems, and computers. While cutting edge research has trended toward the use of genetically encoded calcium or voltage indicators, two-photon microscopes, and in vivo preparations, it is worth noting that some questions still may be best approached using more classical methodologies and preparations. In this review, we highlight a few examples in neurons where the combination of charge-coupled device (CCD) imaging and classical organic indicators has revealed information that has so far been more informative than results using the more modern systems. These experiments take advantage of the high frame rates, sensitivity, and spatial integration of the best CCD cameras. These cameras can respond to the faster kinetics of organic voltage and calcium indicators, which closely reflect the fast dynamics of the underlying cellular events. © 2015 SPIE

Keywords: charge-coupled device cameras; epifluorescence; image acquisition/recording; neurophotonics; signal-to-noise ratio.

1 Introduction
Dynamic calcium and voltage imaging has been a major tool in cellular neuroscience for over 40 years. In that time, there have been major improvements in indicators, microscopes, imaging systems, and computers. Cutting edge research has trended toward genetically encoded calcium indicators (GECIs) or voltage indicators (GEVIs) because of their clear advantage in targeting specific cell types, and use of two-photon microscopes, and in vivo preparations. However, it is worth noting that some questions still may be best approached using more classical methodologies. For example, although recently developed GECIs and GEVIs have greatly improved sensitivity and kinetics compared with the early generation of these probes, and are beginning to be used in physiologically relevant studies they are still not as sensitive as or as fast as some of the popular organic indicators like Oregon Green BAPTA-1 (OGB-1), OGB-5N, and fluo-4 (for calcium) or RH-795, di-4-ANEPPS, and di-2-ANEPEQ (for voltage).

Similarly, two-photon microscopes, light sheet microscopes, and other confocal systems have clear advantages in thick tissue where optical sectioning is important and where the cells of interest may be hundreds of microns below the surface of the preparation. However, if these considerations are not critical, then some modern charge-coupled device (CCD) cameras may offer a combination of speed, sensitivity, and field of view that scanning systems cannot achieve.

In this review, we highlight a few examples of experiments where high-speed CCD imaging and classical indicators have revealed information that has so far been more informative than results using genetically encoded indicators and two-photon systems, and, in some cases, has not even been detected by these newer systems. The Cohen laboratory has been a primary force in developing and exploiting this approach. The newer results described below build on these pioneering experiments.

2 [Ca^{2+}]_i, Measurements in Slices and Culture
One interesting example is the measurement and analysis of calcium release from internal stores. These [Ca^{2+}]_i increases occur in the form of waves, sparks, and puffs whose properties cannot be easily inferred from electrical measurements. In particular, the puffs and sparks occur at unpredictable locations, making it necessary to look over an extended region to enable the detection (Fig. 1). These events are often very fast (rise times less than 10 ms, fall times less than 70 ms) requiring a high-speed detection system and fast responding indicators, especially if the measurement of Ca^{2+} diffusion from the spark source is important. If a neuron in a slice is filled with an organic indicator from a patch electrode and the cell (or part of the cell) is not too far below the surface of the slice, then some of the advantages of two-photon systems are not critical. For example, sections of thin dendrites and axons can be examined using an ordinary microscope and a CCD camera since the neuronal processes are thinner than the depth of focus of either system. In these

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conditions, one advantage of a CCD detector system is that the high-speed images can be made from spatially extended structures while detection at similar speeds with a two-photon system requires that the measurements use a line scan mode, restricting signals to very small areas or straight segments. This property has been useful in the detection and measurement of Ca\(^{2+}\) sparks and puffs in dendrites, and in total internal reflection fluorescence measurements in cultured neuroblastoma SH-SY5Y cells. A line scan across the dendrite cannot be positioned at the right location to detect a spark since that location is not known in advance. Even if that location was known, a transverse line scan would reveal neither the spatial extent of the spark or puff nor the dynamics at different locations. In principle, a scan along a dendritic segment might capture a spark and its spatial extent, but such measurements have not been reported.

Video rate confocal systems have been used to follow [Ca\(^{2+}\)] changes in dendrites, but did not detect sparks or puffs, possibly because the frame rate and sensitivity were not adequate. These considerations would not apply to the detection of a [Ca\(^{2+}\)] change due to an action potential that backpropagated over the dendrites [backpropagating action potential (bAP)] since these signals are almost the same at most locations and, therefore, a transverse line scan is almost certain to detect this signal and to reveal the important physiological information (when and if the spike occurred and the duration of the [Ca\(^{2+}\)] change). One group did use two-photon microscopy to detect

spark-like events in presynaptic terminals, imaging small X – Y regions in the terminal at 100-ms frame interval. Detection with this slow rate was successful in part because the sparks were so long-lasting in the terminal (over 1 s, possibly because a high concentration of indicator was used, which would severely buffer and slow the transients).

Two-photon microscopes and other confocal systems have been used to detect sparks in myocytes and muscle fibers by aligning the scan along the axis of the cell. Line scans along the fiber axis at 500 Hz were able to detect the very rapid (4-ms rise time, 9- to 15-ms fall time) sparks in muscle cells but may have missed some events since myocytes are wider than the scan width. Line scans in oocytes detected some IP\(_3\)–mediated puffs below the surface of the cell, but could not determine where the scans intersected the mass of the [Ca\(^{2+}\)] change and, therefore, could not accurately determine the kinetics and spatial extent of these localized events.

So far, these puffs and sparks have only been detected with organic calcium indicators, whether the detector was a CCD camera or a two-photon system. There are several reasons why these indicators may be preferred for this application compared with GECIs apart from the fact that the GECIs are newer and have not been widely tested. First, the signals are usually small in neurons, typically 20% to 80% of the amplitude of the bAP signal at the same location in the apical dendrites of a pyramidal neuron. Single spike-evoked Ca\(^{2+}\) signals detected with

Fig. 1 Calcium sparks and puffs detected in pyramidal cell dendrites. (a) Image shows a rat pyramidal neuron filled with 50-µM OGB-1. A line of pixels along the dendrite and three ROIs (regions of interest) are shown. The pseudocolor image to the right shows the [Ca\(^{2+}\)] changes along the pixel line as a function of time. There are localized, asynchronous signals at different locations along the dendrites. The colored traces at the bottom show three of these signals at the locations of the ROIs. The black trace at the bottom shows the simultaneously recorded membrane potential from the soma. Sharp transients were detected at each location with no corresponding change in potential (from Ref. 13). (b) and (c) Localized signals in other cells. The “sparks” occur spontaneously. They have fast (∼5 ms) rise times. The traces on the left show the amplitude profile along the line of pixels; the red arrows mark the half maximum of the profile, indicating the spatial extent of the “spark” or “puff.” The “puffs” require IP\(_3\) (either from mGluR synaptic activation or IP\(_3\) uncaging). They are slowly rising and more sensitive to the IP\(_3\) receptor blocker heparin (from Ref. 14).
GECIs are often close to the noise level in many neurons, suggesting that the spark and puff signals would be even harder to detect. Second, the spark signals come from localized regions, making it difficult to spatially average the signal to improve the S/N. In contrast, spatial averaging of the spike signal over the entire somatic region often has been used, especially in in vivo experiments. Third, the spark fluorescence signals are usually faster than spike-evoked signals because the decay time of the spark transient is shortened by diffusion away from the localized source in addition to removal by a pump. Since the kinetics of GECIs are slower than those of organic indicators, the intrinsic speed of the underlying Ca$^{2+}$ transient will lead to filtering of the transients making them harder to detect. A related issue is buffering of the Ca$^{2+}$ transients by the indicator. It is well known that buffering by exogenous indicators slows the fluorescence transients and reduces their amplitude. Because the concentration of organic indicator is known (at equilibrium equal to the concentration in the pipette), the effect of this buffering can be calculated and extrapolated to a zero indicator concentration. In contrast, the concentration of GECIs in cells is not known and their buffering effect has not been determined or corrected for in current experiments. Nevertheless, recent developments with GECIs have shown that they can detect Ca$^{2+}$ signals from small volumes like spines and can detect fast spike-evoked signals, so it may be possible in the future for GECIs to be used in spark detection and analysis.

3 Sodium Imaging

Much less attention has been addressed to follow Na$^{+}$ changes in neurons. Only a few indicators for this ion have been developed and the best one, sodium-binding benzofuran isothiopholate (SBFI), was synthesized over 30 years ago. No genetically encoded indicators for this ion have been developed. All the reported experiments were done in slices or in culture. There are no in vivo studies, probably because the signal size is usually much smaller than with calcium indicators and, in most cases, the goal of the experiments, to optically detect spikes in neurons, can be accomplished easier with calcium measurements.

Fig. 2 Synaptically activated and spike-activated sodium changes in Purkinje cells. (a) The image shows a Purkinje cell in a rat cerebellar slice filled with 2-mM SBFI. Several ROIs are marked. The arrow points to the site of the patch recording. In response to a train of 10 climbing fiber synaptic inputs, [Na$^{+}$]$_s$ changes were detected in the soma and dendrites. The electrical traces are shown at the bottom with the first two responses expanded in the inset. The arrow points to the dendritically recorded Na$^{+}$ spike. The fluorescence changes in the more distal dendrites (regions 5 and 6) were slower than those in the main dendrites (regions 3 and 4) because Na$^{+}$ diffused there from the sites of synaptic contact. (b) Climbing fiber inputs (first five transients) evoke [Na$^{+}$]$_s$ changes all over the cell but fast intrasomatically evoked Na$^{+}$ spikes make changes only in the soma because Na$^{+}$ channels are restricted to this region. Images acquired at 40 Hz (modified from Ref. 26).

Fig. 3 Spike-evoked [Na$^{+}$]$_s$ increases are rapidly rising in the axon hillock but slowly rising more distally in the myelinated region. The image shows the soma and axon of a L5 neocortical pyramidal neuron in a rat neocortical slice. The cell was filled with 2-mM SBFI from the patch electrode on the soma. The Na$^{+}$ signals were acquired at 500 Hz. (a) The signals decay most rapidly near the cell body because of Na$^{+}$ diffusion into the soma. (b) An expanded version of the beginning of these traces. In the initial segment, the signals rise within 1 to 2 ms of the spike. But just a few microns away, the rise time is much slower because there are few Na$^{+}$ channels under the myelin; the signal there comes from diffusion from the initial segment region (modified from Ref. 28).
Fig. 4 Voltage recording from axons. (a) Synaptic stimulation. Upper image: high-resolution confocal image of a stained neuron with axon in recording position. Recording electrode attached to soma and stimulating electrode next to basal dendrite shown schematically. Lower image: low-spatial resolution fluorescence image of the axon obtained by $80 \times 80$ pixel CCD used for $V_m$-imaging. (b) Electrode recordings from soma, optical recordings from spike trigger zone (AIS, red) and from node of Ranvier (green). Top traces: raw data from nine trials showing temporal jitter in AP initiation following synaptic activation. Second row of traces: temporally aligned signals. Third row of traces: averaged signal. Fourth row of traces: bleach correction. Bottom traces: cubic spline interpolation with one pass of temporal smoothing. (c) Intracellular stimulation. Upper image: high-resolution confocal image of another neuron with axon in recording position. Lower image: low-spatial resolution fluorescence image of the axon obtained by CCD used for $V_m$-imaging. Traces on right: AP transients from three locations: (1) electrode recording from soma; (2) optical recording from axon hillock; (3) optical recording from the first node of Ranvier. (d) Superimposed signal from same three locations. Note that the spike narrows as it propagates in axon. Measurement of the spatial distribution of membrane potential as a function of time along the proximal axon during AP initiation. (e) High-resolution confocal image of the axon in recording position. (f) Low-spatial resolution fluorescence image of the axon obtained by $80 \times 80$ CCD used for $V_m$-imaging. (g) AP signals from 10 locations indicated by yellow rectangles in (f), each 10 $\mu$m in length. (h) Soma–axon latency to 30% (gray) and 50% (black) $V_m$-amplitude as a function of distance from the cell body. The first minimum identifies the location and length of the spike TZ. (i) Time sequence of frames showing spatial profile of color-coded relative $V_m$-amplitude in the axon at four characteristic time points: 0 $\mu$s—AP initiation at TZ; 45 and 80 $\mu$s—invagination of the first node; 240 $\mu$s—peak depolarization. (j) Comparison of AP signals from four characteristic locations on an expanded time scale. The measured data points and cubic spline interpolation curves are shown. Red traces—TZ and first node; green dashed trace—axon hillock; green trace—first internodal region. Membrane potential color scale shown on left (from Ref. 38).
Nevertheless, several investigators reported interesting measurements in slices using CCD cameras. Very high speeds were usually not necessary for these early experiments; video rates were adequate to detect the signals (Fig. 1). Since the signal size was small, it was important to optimize all the components in the systems and to use cameras with good quantum efficiency and low noise. In several cases, an important part of the experiment was to detect and interpret the diffusion of sodium away from a localized source. Since Na⁺ diffuses about 100 times faster than free Ca²⁺ in cytoplasm, the physiological consequences of diffusion are different for these two ions. In these experiments, high camera frame rates were important; in some cases, rates of up to 500 Hz were useful (Fig. 1). A few experiments measured [Na⁺], changes in dendrites and spines with a two-photon microscope using line scans to achieve high-time resolution. But these measurements, while informative, did not detect the signals with the same S/N as with CCD cameras and could not resolve synaptically activated subthreshold [Na⁺] changes.

4 Voltage Sensitive Dye Imaging

Understanding the function of individual neurons requires that we measure, analyze, and understand spatiotemporal dynamics of electrical signals. Electrical signals are measured directly and most precisely using electrodes. However, the electrode measurements report voltage changes from a single location on a neuron, while the electrical behavior of other parts of the cell remains uncertain. One can extend intracellular electrode measurements to a few locations but never to tens or hundreds of locations. A second limitation is that small structures like axons and axon collaterals, terminal dendritic branches, and dendritic spines do not tolerate electrodes and, as a rule, cannot be probed for electrical signals. Voltage sensitive dye (VSD) recording techniques (V_dye-imaging) can solve both these problems, allowing multiple site recordings of electrical events from all locations on individual neurons. However, to be effective, these kinds of measurements must use indicators that accurately follow the voltage profile at the recording site and must use a measuring system that has the temporal and spatial resolutions to distinguish important differences in the signals from different locations.

In the first example, high-speed V_dye-imaging with organic VSDs and a NeuroCCD-SMQ camera was utilized to monitor action potential signals from axons in brain slices. The measurements were carried out to determine the location and length of the spike trigger zone and to analyze the pattern of spike initiation and propagation in the axon of layer 5 cortical pyramidal neurons and Purkinje cells of the cerebellum. The type of raw optical recordings and the data analysis procedures utilized in these experiments are illustrated in Fig. 4. From this kind of measurement, it is possible to determine the location and length of the axonal spike initiation site. These two parameters are obtained directly from multisite optical recording of the membrane potential transients either by investigating spike latencies at the soma/axon hillock and more distal axonal recording locations or by the inspection of the spatial distribution of membrane potential as a function of time. These measurements used the NeuroCCD-SMQ camera operating at 10 kHz to achieve the required high-time resolution. In previous experiments, it was established that the electrochromic VSDs track the voltage signals practically instantaneously at the biological time scale (time constant faster than 2 μs). The ability to monitor the spike initiation in the axon initial segment is important because it has been recently discovered that intricate details in the spatial pattern of channel clustering play a critical role in tuning neuronal computations and changes in channel distribution have been shown to mediate novel forms of neuronal excitability in the axon.

In the second example, high-speed V_dye-imaging with a CCD camera in wide-field epi-fluorescence microscopy mode was utilized to re-examine the specific question of the transfer of dendritic electrical signals across spine neck to spine heads. Using the camera enabled the detection from the spine head and nearby dendritic regions simultaneously. The sensitivity and the spatiotemporal resolution of V_dye-imaging were adequate to simultaneously determine the amplitude and the time course of bAP signals in the heads of individual spines and in parent dendrites (Fig. 4). These measurements were carried out in four groups of spines with different neck lengths. The recordings of membrane potential transients from individual spines linked to a computational model provided evidence that synapses on spine heads are not electrically isolated from membrane potential signals in the parent dendrite. It is possible to use two-photon detection of VSD signals from dendritic spines. However, to make these measurements at sufficient speed (over 10 kHz) to follow the voltage transients, the beam could not move and measurements were reported from only a single voxel.

5 Conclusions and Future Prospects

The diversity of currently available calcium and voltage indicators provides opportunities to do many different kinds of
experiments. At the time when only organic indicators were available, the most significant properties that mattered to users were sensitivity, i.e., how good was the S/N for detecting the event of interest, and the kinetics, which reflected how well the indicator followed the time course of the underlying event. Other important properties include the wavelength of excitation and emission and how easy it was to load the indicator into the cell or to stain the preparation. The wavelengths were important because some indicators were used in cells that had light sensitivity and it was useful to excite outside this sensitive region. Indicators with excitation bands at longer wavelengths were also less likely to cause photodynamic damage. In addition, some indicators were used in experiments where other signals were examined and it was useful to have no wavelength overlap in the signals. For calcium indicators, it was also important in some experiments to have enough sensitivity to permit the indicator to be used at a low enough concentration so as not to be a significant buffer. For VSDs, it was important for the indicators to accurately track the voltage change, to have good sensitivity, to have few pharmacological effects, and to cause the minimal photodynamic damage. Many different voltage indicators, sensitive in different wavelength regions, are currently available. For sodium indicators, the available probes are not as diverse. The original probe, SBFI, is still the one used in most experiments, although some new indicators have just become available. Probes are also available to report on the changes in chloride concentration and pH, but these are not extensively used for examining real-time neuronal signaling.

Genetically encoded indicators potentially offer the additional important properties that they can be targeted to specific cell types and to specific parts of a cell, and they can be encoded into the genome, and be transmitted through generations and available for long-term studies. They may also be less toxic to the neurons in which they are inserted, but this property has yet to be examined rigorously. However, the kinetics, linearity, and sensitivity of currently available probes are not yet as good as many organic indicators and this limits the kinds of measurements that can be made. An additional factor is that these probes are not easily titrated in cells; if the concentration is not known, then the buffering or other effects of the indicator cannot be controlled.

So far, the main use of GECIs and GEVIs has been to detect action potentials or other measures of electrical activity in the brain. For these applications, the main requirements are sensitivity and sufficiently rapid kinetics to distinguish individual action potentials. Although there is significant progress in this field, many currently available GECIs do not respond rapidly enough to separate the spikes in a burst, so spike numbers often have been inferred from the amplitude of the signal. This approach is not always accurate. Also, the kinetics are still too slow to accurately follow the removal of calcium in cells or the rise time of the [Ca$^{2+}$], changes in those neuronal compartments where they have been evaluated. Similarly, GEVIs, while far from optimized, have best been used to detect in vivo neuronal activity and subthreshold events in Drosophila. Progress in this field is rapid. An early version of ArcLight had kinetics fast enough to resolve individual spikes in bursts at 30 Hz, while an even newer GEVIs like ASAP1 and ArcLight A173 can follow signals at over 100 Hz. Nevertheless, these probes are still too slow to track the details of spike shape or the propagation of action potentials within individual cells.

It is hard to predict the future of imaging systems since developments are occurring in many different directions. In this review, we have concentrated on the ways in which high-performance CCD cameras can reveal information that is difficult to acquire using two-photon microscopes, while recognizing, of course, the many advantages of multiphoton systems. One advantage of CCD systems is that they can examine spatially extended regions at high frame rates (10 kHz in the examples above). Three groups have developed programmable multisite sampling methods for two-photon imaging that might have the speed to detect the propagation along axons or dendrites. These systems use acousto-optical defectors to position the laser spot to select a number of arbitrary sites for repetitive sampling at 500 Hz to several thousand hertz. These systems can be considered as line scan detectors with arbitrary paths and point separations. So far, they have only been used to measure the spike generation and propagation using calcium indicators. They have not been used to make VSD measurements or to detect and analyze sparks. It is not clear if this kind of sampling system can achieve the S/N that the high-speed VSD recordings require. CCD cameras, which are constantly detecting light at all locations, may be better, especially if signals can be spatially averaged. Another potential limitation is that in this approach, the investigator has to select the sampled points in advance and so might miss critical events at other locations if they are localized and cannot be predicted before the trial. One limitation with both CCD cameras and two-photon systems is photodynamic damage and indicator bleaching. The higher the frame rate and the smaller the sampled voxel, the higher the light intensity required to make a measurement with an adequate S/N, leading to greater damage. This problem has been apparent almost since the beginning of dynamic imaging experiments over 40 years ago. While not all indicators generate the same level of damage, suggesting that the screening for better probes might be useful, and several technical schemes for reducing injury have been tried this still remains a serious problem.

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Dejan Zecevic is a senior research scientist at Yale University School of Medicine. He received a PhD degree in biophysics from University of Belgrade, Serbia, and was trained in the laboratory of Dr. Lawrence Cohen at Yale Medical School and Ann Stuart at Harvard Medical School. His work on electrical and functional structure of individual neurons utilizes intracellular voltage-sensitive dye imaging technique, a unique and a cutting-edge technology for monitoring membrane potential signals in axons, dendrites, and dendritic spines.