Published in final edited form as:
Nat Protoc. 2008 ; 3(2): 249–255.

***In vitro* functional imaging in brain slices using fast voltage-sensitive dye imaging combined with whole-cell patch recording**

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Abstract

In many brain areas, circuit connectivity is segregated into specific lamina or glomerula. Functional imaging in these anatomically discrete areas is particularly useful in characterizing circuit properties. Voltage-sensitive dye (VSD) imaging directly assays the spatiotemporal dynamics of neuronal activity, including the functional connectivity of the neurons involved. In spatially segregated structures, VSD imaging can define how physiology and connectivity interact, and can identify functional abnormalities in models of neurological and psychiatric disorders. In the following protocol, we describe the *in vitro* slice preparation, epifluorescence setup and analyses necessary for fast charge-coupled device (CCD)-based VSD imaging combined with simultaneous whole-cell patch recording. The addition of single-cell recordings validates imaging results, and can reveal the relationship between single-cell activity and the VSD-imaged population response; in synchronously activated neurons, this change in whole-cell recorded V_m can accurately represent population V_m changes driving the VSD responses. Thus, the combined VSD imaging and whole-cell patch approach provides experimental resolution spanning single-cell electrophysiology to complex local circuit responses.

INTRODUCTION

Compared to single-cell electrophysiology, our ability to directly study neuronal circuit function has been limited. Much of our perception of local circuit function in the brain arises from the extrapolation of neurophysiological data derived from single neurons and their synapses into their anatomical connectivity. These data are invariably incomplete, and frequently underestimate or completely disregard emergent properties of neurons functioning in ensembles. Imaging function in slices is therefore attractive, particularly because in many brain areas, local circuit connectivity is segregated into specific lamina or glomeruli (e.g., hippocampus, neocortex and olfactory bulb). Imaging activity in slices, using fast voltage-sensitive dyes, was pioneered using high temporal but low spatial resolution photodiode arrays^{1–6}, although for slower signals, higher resolution video cameras were also developed⁷. Using VSD imaging to probe neuronal circuitry function has continued over the last decade with increasing sophistication as the technology has matured; yet widespread use

has been limited due to the effort and the mix of technical expertise necessary to run successful imaging experiments.

Fortunately, the field has advanced so that VSD imaging can now be a part of the electrophysiologist's toolkit. Introduction of fast (and more affordable) CCD and complementary metal-oxide-semiconductor (CMOS) cameras have facilitated optical imaging of neuronal circuit function. One issue or limitation is that individual pixels have relatively high noise, higher than what is typical of photodiodes used for VSD imaging. Because noise on an individual pixel is high, averaging over a number of pixels in 10–14 trials is sometimes necessary. The other dimension to average is space, and this is where the advantage of a large number of pixels is most apparent, because anatomical boundaries can be easily identified and pixels can be grouped for averaging by their anatomical location. Thus, these cameras significantly increase the spatial quality of the data over photodiode arrays. These raw data provide a substrate from which it is possible to extract information from anatomically discrete regions of interest, while maintaining temporal resolution to rival electrophysiology (1–10 kHz). In combination with enhancement in functional properties of VSD, including reduced phototoxicity and internalization⁸ and the reduced light levels used during CCD imaging, these cameras also make possible longer recording periods with intact physiological responses (we have run hundreds of trials over hours without apparent change in physiology⁹). Finally, the ability to routinely conduct simultaneous current-clamp recordings from neurons can facilitate the correlation of the VSD signals from a population of cells to activity in a single cell^{9–12}.

VSD images from slices record responses from areas encompassing multiple neurons and/or processes, and so VSD signals are often compared with field potentials; however, they are not analogues. VSD signals, unlike field potential recordings, record voltage changes in neuronal membranes; thus, they provide signals more analogous to responses recorded in whole-cell current-clamp mode. VSD imaging can record the spatiotemporal dynamics of both dendritic integration and inhibitory postsynaptic potential (IPSP) activation, phenomena that are difficult or impossible to capture using most other physiological recording techniques. Because of the high spatial and temporal resolution of the activity recorded by CCD-based VSD imaging, the raw data provide a substrate with which it is possible to visualize where voltage changes occur, how large these events are and how they move through the slice in time. This multidimensional recording (x and y distance, time and voltage) is critical in understanding synaptic integration in circuits.

VSD imaging also records voltage changes from processes too small to access with patch-clamp electrodes in intact cells. Nevertheless, successful VSD recordings from slices require many of the same skills and present many of the same challenges as high-quality single-cell electrophysiology in slices^{13–15}. For instance, slice health is paramount. In VSD imaging, the investigator usually images from above using an epifluorescence microscope (Fig. 1). Slices that show little surface damage and a large number of healthy cells when visualized under high power in a submerged visualized patch setup will provide the best recordings under the lower power objective in the VSD setup. Because the VSD signals can be very small, movement produces significant optical noise. Therefore, to an even greater extent than when conducting whole-cell patch-clamp recordings, mechanical vibrations must be minimized, as movement anywhere along the light path will produce changes in intensity.

Because of the similar needs of electrophysiology and successful VSD imaging, the VSD imaging setup can provide a good platform for whole-cell patch recording in the blind-patch mode, as shown in Figures 1 and 2. Although visualized whole-cell patch has eclipsed blind patch for recording from neurons in slices, the Blanton blind-patch technique can still be a very efficient method, particularly in laminar cortical structures such as the hippocampus^{16,17}. This

patch technique is well suited for conducting cellular recordings in slices imaged in the VSD recording setup, because it works well in slices kept in interface conditions and there is no need to change from a low to high power objective for visualizing a cell to be patched. Particularly when imaging populations of synchronously activated and stereotypically arrayed neurons, as found in the laminar fields of the hippocampus, single-cell current-clamp recordings can validate the VSD results by demonstrating that similar kinetics can be found for both whole-cell and VSD-recorded population responses (Figs. 1 and 2). If the kinetics of each response are similar, it is useful to ask how the amplitudes of the responses scale. As shown in Figure 1, a relatively consistent relationship between changes in membrane voltage (ΔV_m) as recorded in single cells to changes in normalized fluorescence ($\Delta F/F$) can be demonstrated in area CA1. Although it is likely that other locations outside the dendritic responses illustrated in Figure 1 would have differing $\Delta F/F$ to ΔV_m relationships, these simultaneous recordings provide a useful calibration of fluorescent changes to ΔV_m . Equally important is the fact that simultaneous patch brings the whole-patch repertoire to bear, facilitating association of synaptic and intrinsic neuronal properties of individual cells with circuit activity imaged using fast-VSD techniques.

MATERIALS

REAGENTS

- Experimental animals: mice or rats ! **CAUTION** Experiments using live rodents must conform to local and national regulations.
- Sucrose artificial cerebral spinal fluid (sACSF) and normal (NaCl) ACSF (nACSF; see REAGENT SETUP)
- Intracellular solutions for whole-cell patch clamping (see REAGENT SETUP)
- Voltage-sensitive dye: RH795 (Invitrogen) or JPW 3031 or JPW 5029 (Leslie Loew and Joe P. Wurrel, University of Connecticut Health Center). Recent advances in dyes sensitive to longer wavelengths, such as RH1691 (see ref. ¹⁸) (Optical Imaging) and JPW 3080 (see ref. ¹⁹), may also be useful
- Tea bag paper or similar fine paper for supporting individual slices
- $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Sigma)
- D-Glucose (Sigma)
- EGTA (Sigma)
- HEPES (Sigma)
- KCl (Sigma)
- K-gluconate (Sigma)
- KOH (Sigma)
- Mg-ATP (Sigma)
- $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Sigma)
- NaCl (Sigma)
- Na-GTP (Sigma)
- $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (Sigma)
- NaHCO_3 (Sigma)

- Agar (Sigma)
- Carboxygen (95% O₂/5% CO₂)

EQUIPMENT

- Dissecting tools for removing brain (e.g., scissors, fine forceps, rongeurs for adult rats, etc.)¹³
- Static interface chamber for recovery and staining of brain slices (see EQUIPMENT SETUP)
- Upright, fixed-stage microscope configured for epifluorescence illumination (see EQUIPMENT SETUP)
- Stable light source (e.g., Optiquip 200 W xenon with light guide)
! CAUTION VSD signals are extremely small (0.05%–0.3% in our hands with 0.1% change approximating 10 mV change in V_m); every effort should be made to reduce optical noise. The power source, lamp or various mechanical sources can all generate optical noise.
- Haas top interface chamber customized for microscopy by removing the typical bottom chamber and replacing it with an oxygen humidifier located off the stage (Warner Instruments)
- Fast CCD camera (NeuroCCD-SM provides an 80 pixel × 80 pixel capture at up to 2 kHz without binning, and up to 10,000 kHz with binning; RedShirtImaging). Other cameras for slice VSD include the CMOS-based MiCAM ULTIMA and NeuroCMOS-SM (RedShirtImaging)
- Whole-cell patch amplifier with good current-clamp abilities (e.g., Axon Instruments Axoclamp-2B or Multiclamp700B; Molecular Devices)
- Digitizing equipment and software must provide for continuous resistance testing and display for blind patch as well as concurrent inputs for synchronizing imaging and electrophysiological data
- Pipette puller appropriate for patch electrodes (e.g., Sutter P series Flaming/Brown pipette puller; Sutter Instruments)
- Micromanipulators and pipette holders for patch clamping and placing stimulation and field electrodes (e.g., Sutter Instruments, Burleigh and Narishige)

REAGENT SETUP

Extracellular solutions—For postdissection recording and slice recovery, we use nACSF comprising of (in mM) 130 NaCl, 3 KCl, 1.25 NaHPO₄, 1MgCl₂, 2 CaCl₂, 26 NaHCO₃ and 10 dextrose. For cutting, NaCl is replaced by equiosmolar sucrose (sACSF). At all points, it is essential that the ACSF in contact with the brain tissue be saturated with carboxygen (which provides both oxygenation and pH buffering). Final osmolarity should be 305 mOsm. This solution should be prepared fresh each day.

Internal patch-pipette solution for whole-cell recording—Comprising of (in mM) 145 K-gluconate, 2 MgCl₂, 2 Mg-ATP, 0.5 GTP-Tris, 0.1 BAPTA (1,2-bis(*O*-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid), 2.5 KCl, 2.5 NaCl and 10 HEPES titrated with KOH to pH 7.2. Final osmolarity should be 285 mOsm (see TROUBLESHOOTING). Aliquot the final solution into 1 ml microcentrifuge tubes and store frozen at −80 °C until use.

Dye stock solution—If using RH795, dilute a 1 mg vial in 20 μl ddH₂O and use as the stock solution. Final concentration of the dye solution used in slice staining should be between 0.1 and 0.2 mg ml⁻¹ in nACSF, freshly prepared on the day of the experiment. Although increasing the dye concentration to enhance VSD signal is tempting, higher dye concentrations are associated with higher phototoxicity and nonspecific staining, which may degrade the $\Delta F/F$ signal.

EQUIPMENT SETUP

Static interface chamber for recovery and staining of brain slices—This can be fashioned from a box for 1,000 μl pipette tips modified so that carboxygen can be humidified by bubbling through distilled water at the bottom of the chamber. On the area that the pipette tips are usually stored, place a pair of 20 ml Petri dishes filled with fresh, oxygenated nACSF and covered with filter paper (Whatman no. 1, 22 mm round). Place slices resting on tea bag paper on the nACSF-saturated filter paper. Care should be taken that water droplets do not fall on the slices.

Microscope—Light source and shutter should be mechanically isolated from the microscope and stage. Even with isolation, using a pretrigger to open the shutter 200 ms before stimulation will allow any residual vibration associated with shutter function to subside before the onset of VSD recordings. This will further reduce mechanical noise. Other potential stable light sources include tungsten filaments and light-emitting diode (LED)-based illumination. Tungsten filament can produce a larger and more stable illumination source, whereas LEDs can be turned on and off extremely quickly (<1ms), eliminating the need for a shutter. In addition, LEDs can be very efficient, with most of the energy produced in a narrow spectrum, and are very stable over long time periods²⁰.

The camera for VSD imaging should be mounted in the cleanest light path (usually top camera port above a trinocular head). Because these cameras often have nonstandard chip sizes, care should be taken in selecting objectives and camera couplers to match the area viewed onto the camera chip. Although not absolutely necessary, an additional camera port and camera for taking high-resolution images is useful for both analysis and presentation.

For simultaneous patch recording, a fixed-stage microscope should be used, mounted on an xy-translation stage. Because the working distance of even low-power, high-numerical-aperture objectives is limited (e.g., Olympus XLFLUOR · 4/340 with a numerical aperture of 0.28), a low angle of approach for the patch pipettes (15–25° from horizontal), similar to submerged visualized patch, is necessary.

For recording, all solutions and humidified carboxygen should be heated to 34 °C. Dead volume of the perfusion system should be limited by using small bore tubing and limiting tubing length between reservoir and recording chamber. It is necessary to maintain a humid oxygen-rich environment above the slice. Coverslips, or a custom lid built to limit dissipation of the humidified gas environment yet allowing recording, can be used (see TROUBLESHOOTING).

Upright, fixed-stage microscope—Low-power ($\times 2$, $\times 4$, $\times 10$ or $\times 20$) objectives are necessary to image a local area such as CA1, but, to ensure adequate epifluorescence transmission, objectives with a high numerical aperture are usually optimal. To separate excitation and emission signals, a filter cube is necessary (Fig. 1). For dyes RH795 and JPW3031, we use a cube containing an excitation band-pass filter centered at 535 nm and 50 nm wide, a high-pass emission filter with a 590 nm cutoff and the emission path is separated from the excitation path by a dichroic mirror centered at 565 nm (Wide Green; Chroma Technology Corp, cat. no. 11007v2).

Micromanipulators and pipette holders—A good quality, high-resolution and stable manipulator is required for patch recordings. This manipulator is usually controlled remotely, using either motors or hydraulics. Many companies, including Sutter Instruments, Burleigh and Narishige, make patch manipulators of acceptable quality. Field and stimulation electrode manipulators can be of lower grade, and do not need to be controlled remotely.

PROCEDURE

Cutting

1. Prepare for cutting by making sure that all portions of equipment and tools that will touch the brain are ice cold. Depending on the portion and angle of the brain slice, agar blocks to support the brain during cutting will likely be necessary. For making hippocampal slices maintain connections with the entorhinal cortex, the medial portion of both blocked hemispheres needs to be supported by an approximately 1 cm cube of agar and the rostral end of each hemisphere needs to be supported by ramps of agar that will elevate it 12° off a horizontal plane. Glue these to the chuck of a vibrating blade slicer and place the chuck on ice (or cool it otherwise).

2. Anesthetize the animal and quickly decapitate it. Uncover the brain using sharp scissors and rongeurs. The specific technique depends on the age and species of the animal, and also on the part of the brain to be imaged. For imaging hippocampus in adult rat, make a cut up the midline from the back of the skull. Capture the edges of the cut bone by one blade of open rongeurs and pry up off the brain. Then quickly remove the brain with a small, sharp spatula blade or small scissors and place it in ice-cold sACSF.

▲ **CRITICAL STEP** The brain must be removed in 60 s or less, without undue compression or damage to tissue. Once removed and chilled more time can be taken as the chilled brain has lower oxygen requirements.

3. Block the brain for cutting and glue to the chuck or agar blocks. Cut slices of 350–400 µm in ice-cold sACSF.

▲ **CRITICAL STEP** The brain must be kept ice-cold throughout this process; blocking of the brain must be conducted in ice-cold ACSF or on an ice-cold surface saturated with ACSF. The agar blocks should be glued on the chuck before blocking and the chuck kept on ice.

4. For small slices, capture slices directly on tea paper squares. For larger slices from adult rat, remove and transfer to a Petri dish of ice-cold ACSF to be further blocked under microscopic visualization before being placed on tea paper, and transfer to a holding chamber.

5. Allow the slices to recover in the chamber for 45 min.

▪ **PAUSE POINT** Following recovery, these slices can be used for at least 6 h.

6. Make fresh staining solution by adding stock VSD solution (e.g., JPW3031, 2 µl) to 400 µl oxygenated ACSF in a 1.5 ml microcentrifuge tube. Keep in the recovery chamber exposed to carboxygen to maintain oxygen and pH levels of the dye solution.

7. To dye the slice, pick up a single slice via its tea bag paper support with forceps and place in a dry Petri dish; then immediately cover it with an approximately 80 µl drop of the dye solution for 10–15 min. After staining, remove the slice from the static chamber and place it in the interface recording chamber. Adjust positioning to limit the possibility that electrodes will interfere with the light path by crossing over areas of interest, and to provide a cell-rich path for patching.

Data capture

8. Place stimulus- and field-recording electrodes depending on the response desired and pathways of interest. For hippocampal area CA1, we have looked at interactions between the Schaffer collaterals, temporoammonic and local feedback inhibitory pathways by stimulating, respectively, in stratum radiatum on the area CA1-3 border, in the subiculum on the border of the dentate fissure and alveus of area CA1. Because field electrodes are used primarily for setting up the experiment, for troubleshooting and to providing a continuous assay of slice health, they can usually be placed unobtrusively near the edge of the field.

9. Allow recovery of slice before starting data capture. Stability of evoked responses can be tested with the field electrode.

10. Capture data by averaging 10–14 trials of an evoked response. If desired, these trials can be interleaved with non-stimulus trials to subtract the effects of dye bleaching, or bleaching can be subtracted *post hoc* as part of the off-line analysis (Fig. 2)⁹.

Simultaneous blind patch

11. Fill the pipette with intracellular solution, place it in the holder and align the manipulator to advance the pipette through the area of interest (e.g., along or below the cell body layer in CA1). For calibration of VSD signals to single-cell voltage responses in CA1, we typically record from dendrites.

12. Place positive pressure on the back of the pipette by mouth or via an approximately 0.5 cc displacement from a 5 ml syringe that is connected through a stopcock and tubing to the electrode holder. Visually advance the pipette until it enters the solution perfusing the interface chamber (ACSF will be sucked up over the tip of the electrode and resistance will fall, revealing the pipette resistance).

13. In voltage-clamp mode, monitor pipette resistance on the oscilloscope, or through software (e.g., seal test in pClamp, Molecular Probes), and advance the pipette along its axis into the tissue. Contact of the electrode tip with cellular membrane will generate a 20–40% jump in resistance that will increase with slight forward advancement of the electrode. At this point, immediately remove positive pressure. This should produce a dramatic increase in resistance, indicative of seal formation. If there is no increase in resistance upon removing pressure, we have had success backing up the electrode, reintroducing positive pressure, testing for the original pipette resistance and attempting another patch. Otherwise, replace the pipette and repeat. If there is a jump in resistance, hyperpolarize the pipette to approximately –70 mV using a voltage command, and begin to apply slight negative pressure, with the goal of a gigaOhm seal. Often, it is useful to back up the electrode very slightly to reduce strain produced by pushing on the membrane. Failure at this point necessitates a new electrode.

14. Once a gigaOhm seal is made, break through the membrane by slowly increasing negative pressure, which must be removed immediately upon occurrence of a decrease in resistance. Breakthrough is best performed in voltage-clamp at the expected membrane potential. If your amplifier does not cleanly handle the transition from voltage-clamp to current-clamp (i.e., cells are frequently lost during the mode switch), breakthrough can be performed in current-clamp mode.

15. Identify cells electrophysiologically by passing positive- and negative-current steps to characterize firing properties and voltage-dependent responses.

16. For calibration, record evoked voltage responses at rest simultaneously with imaging. Perform actual calibration off-line, by correlating voltage and fluorescence change for each frame over an area adjacent to the tip of the electrode.

? TROUBLESHOOTING

○ TIMING

Steps 1–6, cutting and recovery of slices: 1 h

Step 7, staining: 15 min

Steps 8–16, experimental period: 8 h

Note: Compared with submerged slices, where solution changes can take 5 min, complete wash-in of a drug can take up to 30 min in an interface chamber.

? TROUBLESHOOTING

Lack of any evoked signal

The most common reason for finding small or no VSD signal is that the slice is damaged (see below). Nevertheless, even in dead slices, fluorescence changes should be seen at the stimulation electrodes. Particularly, in troubleshooting a new setup, if there are no focal fluorescence changes at the stimulation electrode, the problem may be in the stimulation or mistiming of the stimulation and the VSD recording. Other technical possibilities include use of wrong filter sets (not imaging fluorescence wavelengths of the dye that is sensitive to voltage changes) or noise so large that it floods out the signal (see section under Noise).

Intense fluorescence, large evoked potentials, but small VSD signal

It is common, when optimizing a VSD setup, that slices appear healthy and healthy evoked potentials can be produced, but very small or no VSD signals are produced. First, it should be realized that VSD signals are already very small—measured in one-tenth of percentage change. Second, the size of the signal is exceedingly dependent on the proportion of dye bound to active neuronal membrane. Dye bound to dead or depolarized cell membrane or internalized into intracellular membrane will produce a strong fluorescence baseline signal, but will show little response to evoked stimulus. This can be due to staining for too long or in too high a concentration of dye, damage to the slice during preparation or drying of the surface due to problems in perfusion or insufficient moist carboxygen. The latter issues are particularly pernicious as epifluorescent imaging biases for signals arising from the surface of the slice, where blind pipette field and whole-cell-based electrophysiology can and often assays signals from cells deeper in the slice and thus are less sensitive to surface effects.

Condensation on the objective

Because the protocol described here uses an interface chamber, where it is necessary to maintain the slice under warm, humidified carboxygen, condensation on the objective is a frequent problem. Using a long working distance objective as well as limiting the flow of gas to the minimum can help limit condensation on the objective face. This solution is usually adequate; if not, increasing the room temperature or warming the objective with an objective warmer (e.g., Warner Instruments OW series) or both, so that the objective temperature is equal to or greater than the local recording environment, usually minimizes condensation.

Noise

We have found that most noise is mechanical and can be ameliorated by careful isolation of the microscope and tabletop from vibration sources, particularly the mechanical shutter. In some cases, an aging bulb can also produce 60 Hz noise. This type of mechanical noise can be readily identified because it scales with fluorescent intensity, or, in other words, it is similar in all pixels after normalizing to df/f_i . Noise that is similar in all pixels before normalization is likely mixed into the signal as instrument noise, and often arises from a ground loop affecting the camera or its acquisition board, although high carboxygen flow rates can also produce low frequency (>10 Hz) fluctuations. The remaining high-frequency noise is likely ‘shot noise.’ Increasing light levels to drive the emitted fluorescence signal to near-saturation levels of the camera will help reduce the shot noise relative to signal^{4,21,22}.

Difficulty in making seals or maintaining whole-cell patch recordings

Beyond mechanical issues (pipette drift and vibration), osmolarity of the intracellular solution is critical. Because whole-cell sealing occurs more readily at relatively lower osmolarity and cells appear more stable at higher osmolarities that approach the extracellular osmolarity, osmolarity needs to be adjusted in response to the experimenter’s patching success to optimize cell yields in the whole-cell patch portion of the experiments.

ANTICIPATED RESULTS

VSD imaging can provide clear images depicting the spatiotemporal dynamics of the initiation, integration and subsequent propagation of an evoked response within a synaptic circuit (Fig. 2). Analysis of discrete areas of interest reveals that these responses express the familiar kinetics of compound postsynaptic potentials and respond similarly to pharmacological manipulations. For instance, as shown in Figure 1, local stimulation of Schaffer collaterals onto area CA1 neurons of the hippocampus produces a signal that is identical in kinetics to the signal recorded in current-clamp from a single pyramidal cell. This includes both a fast excitatory postsynaptic potential and a hyperpolarizing IPSP. In fact, VSD imaging is very useful for examination of responses that are primarily inhibitory (Fig. 3). IPSPs are absent from field potential recordings, and the spatiotemporal dynamics of IPSP activation and propagation cannot be resolved using single patch recordings. This makes VSD recording an optimal technique to examine inhibitory circuitry in laminar structures, such as the hippocampus, cerebellum and neocortex.

Because noise on an individual pixel is high, averaging over a number of pixels in 10–14 trials is sometimes necessary. Another dimension to average pixels in is space, and this is where the advantage of a large number of pixels is most apparent, as anatomical boundaries can be easily identified and pixels can be grouped for averaging by their anatomical location. These results can be extracted from the raw VSD data with the acquisition software bundled with the Redshirtimaging and Brain Vision cameras. In our laboratories, further quantitative work is most often performed using in-house algorithms written in Igor Pro (Wavemetrics), IDL or Matlab. Nevertheless, in most cases, we find that a single trial or pixel produces a clearly discernable signal¹¹. In fact, in most experiments, visible activity in a single trial is used as our standard for slice health, stimulation electrode quality and placement.

In areas like the hippocampus proper and dentate gyrus, where the bulk of the neurons are principal cells and arranged into lamina, single-cell voltage recordings from dendrites can be used to calibrate change in voltage (ΔV_m) with change in fluorescence (ΔF). These calibration results are reproducible ‘within the same experimental condition,’ but not necessarily between treatment groups. For instance, in the pilocarpine model of epilepsy, epileptic animals show consistently lower $\Delta F/\Delta V_m$ (see ref. ¹¹). Thus, to attribute subtler changes associated with

experimental treatment to changes in voltage response amplitude, it is critical to simultaneously sample slices with whole-cell patch recordings to identify these differences.

Acknowledgements

We thank Brian M. Salzberg and the reviewers for critically reading the paper and for useful comments. We also thank Chyze-Whee Ang for his part in developing these techniques. NIH NINDS Grants NS-32403 and NS38572 supported this work.

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Figure 1.

VSD setup, validation and calibration of whole-cell current-clamp recordings (I-clamp recording) with VSD response. The epifluorescent-based imaging setup is illustrated in (a). The slice is placed in an interface chamber under a long working distance objective, allowing access to the slice for stimulating and recording electrodes, including whole-cell patch electrodes. The slice is illuminated by an intense (200 W xenon) light source that is directed through a dichroic filter cube. The emitted fluorescence signal is then projected onto the CCD chip for acquisition. To limit bleaching and phototoxicity, a shutter is placed at the light source (not shown) and triggered to open 200 ms before acquisition. Images are typically acquired into memory and then transferred to a disc drive for analysis (though direct writing to disk is possible with some cameras, including the NeuroCCD by RedShirtImaging). Validating that the VSD response is predominantly measuring voltage change in principal cells is demonstrated in (b), where an evoked response recorded by VSD (red trace) is overlaid on a dendritic I-clamp recording from a CA1 pyramidal cell. The VSD response is averaged over a small region of interest adjacent to the tip of the electrode, which is patched onto the dendrite. Similar kinetics of the scaled VSD image and the simultaneous I-clamp recording (black trace) is evident in B. With the simultaneously acquired data, we can plot change in fluorescence with respect to change in voltage for each point acquired by the camera (c). The resultant linear relationship, over a physiologically relevant scale (-5 to $+30 \Delta V_m$), demonstrates that whole-cell recordings can be used to calibrate the relationship of fluorescent change to transmembrane voltage deviations. Over a population of cells, this relationship clusters around approximately 0.1% df/f_i for each 10 mV voltage change (d); thus, even in areas or slices without direct whole-cell recordings, we have a measure of the magnitude of the voltage response (similar results in a larger number of cells is published for the dye RH795) (see ref. ⁶). *Neuro CCD-SM, RedShirtImaging, Decatur, GA.

Figure 2.

Analysis and display of VSD data. The gray scale image displayed in (a) shows a single frame from the RedShirtImaging camera. As shown in this image, anatomical features produce relatively large differences in intensity. To identify changes in voltage, analysis begins by normalizing the data by subtracting a reference frame (f_i) from all other frames (delta f , or df), followed by division by the reference frame (df/f_i). Because of the small signal size presently inherent in VSD imaging, noise and dye bleaching during recording produce a strong nonspecific component of the raw signal (b). Averaged response of pixel intensities over time from a region of interest (ROI) encompassing a portion of the dentate blade (raw DG) as well as a trace from the inactive, unstimulated neuropil outside of the hippocampus (Raw bkg) shows a strong exponential decay of fluorescence, and, in this case, the presence of mechanical oscillations in the first quarter of the trace, probably due to residual vibrations from the shutter opening. Because both the bleaching and the effects of vibrations scale with intensity, the most effective way to remove them is to subtract a background signal from an area unaffected by the stimulus (b; bkg). When this is not possible, subtraction of an exponential fit to the baseline period (b; Exp fit; in red) of the recording, or subtraction of an interleaved non-stimulated control recording will also correct for bleaching. To match I-clamp conventions (c), signals

are usually inverted so that a depolarization is upward (dye signal intensity decreases as membrane potential depolarizes). Because the imaging data are collected as a set of 2D images over time, enhancing characterization of various spatial, voltage or temporal aspects of the recording requires that data display be reduced from 4 dimensions (space (x and y), time and voltage) to 2 or 3 dimensions. 2D traces (voltage change over time) as shown in **(b)** and **(d)** are familiar to electrophysiologists and, by averaging, many pixels can show a strong signal to noise. However, similar changes in amplitude over time can also be represented in 3D raster scans as shown in **(e)**. In a raster scan, changes in VSD signal along a line, for each time point, reveal changes in voltage (high intensities are red, and a color look-up table provides the actual intensity), with the addition of an axis coding for space. Using this display, where activity occurs along that line over time and how it propagates can be easily visualized. To show the spatial extent of a response, changes in fluorescence from baseline at a specific time point can also be displayed in the same orientation as the gray scale image **(f)**. For this type of a 3D snapshot display, at the peak of the first response, activity is clearly limited to the dentate gyrus.

Figure 3.

Unlike other electrophysiological measures of neuronal population activity, VSD imaging can directly measure the spatiotemporal dynamics of inhibitory responses by reporting hyperpolarization. This is dramatically demonstrated by an alveus stimulation that activates output axons arising from area CA1 pyramidal cells, which in turn activate feedback interneurons and generate a strong feedback IPSP. Action potentials generated by this stimulation propagate back to the neurons (producing a depolarization; red in **(a)**), but also via axon collaterals to excitatory synapses that impinge onto local inhibitory neurons. Activating these neurons produces the equivalent of feedback inhibition throughout area CA1, producing a hyperpolarization (blue in **(b)**). The spatiotemporal dynamics of this response can be described purely spatially as in **(a)** and **(b)**, but can also be portrayed by comparing traces averaged over ROIs from specific anatomical areas **(c)**. These inhibitory responses from intact neurons can show different kinetics than records from I-clamp recordings (dendritic I-clamp; **(c)**), as IPSPs are very sensitive to internal anion concentrations (perturbed in whole-cell patch) and holding potential. As many signal transduction pathways underlying synaptic plasticity are also lost during whole-cell patch recordings, the potential for efficiently studying neuronal circuitry with the neuron's intracellular milieu and therefore these transduction pathways intact is another important feature of VSD imaging with great promise.