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Imaging of Brain Slices with a Genetically Encoded Voltage Indicator

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Summary

Functional fluorescence microscopy of brain slices using voltage sensitive fluorescent proteins (VSFPs) allows large scale electrophysiological monitoring of neuronal excitation and inhibition. We describe the equipment and techniques needed to successfully record functional responses optical voltage signals from cells expressing a voltage indicator such as VSFP Butterfly 1.2. We also discuss the advantages of voltage imaging and the challenges it presents.

Keywords

Voltage imaging; fluorescence imaging; brain slices; voltage indicators; fluorescence microscopy; voltage sensitive fluorescent proteins

1. Introduction

A key goal of neuroscience is to understand how spatiotemporal patterns of membrane voltage in sets of neurons can encode and compute neuronal information. Classical electrophysiology has enabled great advances in our understanding of cellular functions; however as an essentially one-dimensional technology recording a single time course per electrode, it suffers from a trade-off between recording fidelity, cell number and spatial localisation. Patch clamp electrophysiology can monitor and perturb membrane voltage from a single cell exquisitely accurately [1]; multi-electrode arrays are able to record extracellular potentials from many cells simultaneously but with limited spatial resolution [2]. Optical imaging offers an increase in dimensionality compared to electrical recordings with much improved spatial field of view and resolution at the cost of relying on indicators to transduce neuronal activity into an optical signal. These can be organic fluorescent dye or genetically encoded fluorescent protein-based indicators of membrane voltage or calcium concentration [3]. The indicator kinetics and optical properties determines the type of activity monitored and the achievable spatiotemporal resolution [4].

Calcium indicators are often simpler to use than voltage indicators for a variety of reasons. It is possible to fit a greater number of indicator molecules into the cytosol rather than into the plasma membrane where voltage indicators must be located, increasing the signal brightness. The underlying signal transduced by calcium indicators, transient calcium influx

following action potentials (APs), has slower dynamics than voltage signals, giving the indicators longer to respond and relaxing the lower bound on the required sampling rate. AP-related calcium signals also essentially consist of all-or-nothing events that are easier to extract from noisy data than graded voltage signals. This feature reduces the constraints on the quality of the imaging system.

Calcium signals convey less of the richness of neuronal activity compared to voltage signals. Calcium transients following APs do not occur in all neurons [5], and in some neurons calcium signals are not well correlated with AP firing [6]. Calcium indicators are effectively second order indicators of the neural signal of interest; they transduce a proxy for neuronal activity into an optical signal. Using calcium and voltage indicators in concert could allow large scale analysis of neuronal populations as ‘black box’ computational groupings, with the indicators showing postsynaptic potentials (inputs to neurons) and APs (output of neurons) respectively.

Optical imaging is limited in depth by scattering in tissue. One photon wide-field imaging can image cell-size structures not deeper from the tissue surface than 100µm whilst two-photon imaging can resolve cells as deep as 1mm [7]. To study subcortical structures in the mouse it is therefore necessary to either image through an implanted device (e.g. fibre bundles [8], GRIN lenses [9] or prisms [10]) or turn to imaging of *ex vivo* brain slices. Imaging of brain slices not only offers an opportunity to investigate specific neural circuits that are not easily accessible to *in vivo* imaging methods but also offers manipulations that are difficult under *in vivo* conditions (e.g. addition of pharmacological agents, mechanical cutting of connections). In the context of the approaches described here (development of genetically encoded indicator imaging), slices are also a valuable test bed due to the lack of haemodynamic signals and movement artefacts associated with *in vivo* recordings which have to be accounted for in post-processing [11].

Whilst two-photon imaging is intrinsically optically sectioning and so can image with cellular resolution deep in scattering tissue [12], in its standard implementation it suffers from the fact that by design a single small volume of tissue is imaged at each time point. This volume is then scanned through the sample to build up a 2- or 3-dimensional image point by point. In order to image at rates high enough to resolve neural activity the dwell time at each pixel must be very short (or the number of pixels must be very few). This limits the number of photons that can be collected, reducing the achievable signal-to-noise-ratio (R, see note 1). For calcium imaging, acceptable R values can be more readily achieved due to the typically larger $\Delta F/F$ values; for voltage imaging, where $\Delta F/F$ is typically small, maximising R is a major challenge. One-photon imaging is limited in its ability to resolve single cells in scattering tissues except in special cases. This is because fluorescence excited outside of the focal plane is collected at the camera. This problem can be minimized by sparse labelling of cells, reducing the fluorescence contributed by unfocussed sources.

One-photon wide field imaging is able to collect many more signal photons than two-photon point-scan imaging as photons are collected for all pixels simultaneously. Moreover, one-photon excitation cross sections (i.e. light extinction coefficients) are typically much larger than 2p cross sections. For these reasons, one-photon wide field imaging is more suitable to

resolve small changes in indicator brightness. This feature suits imaging of population activity with indicators such as VSFP Butterfly [13].

In this chapter we describe the equipment and methods needed to image optical voltage signals in mouse brain slices using VSFPs. The protocol we describe is designed to enable imaging of large areas of brain slices at high (100Hz or greater) frame rates and is mainly used to characterise the performance of genetically encoded voltage indicators (GEVIs). It is also well suited to studying communication between different brain regions by imaging the spread of population activity. In conjunction with sparsely expressed indicators activity at the level of single cells could also feasibly be resolved. The set-up is based around commercially available components with custom-written Matlab scripts for data acquisition and analysis.

2. Materials

Microscope

- 1 THT Macroscope (Brainvision Inc.) with the following components:
 - 10x/0.3NA water dipping objective (Nikon)
 - 2x Planapo 1x objectives (Leica)
 - 580nm long pass emission dichroic (Semrock)
 - 495nm long pass excitation dichroic (Brainvision)
 - 542/27nm emission filter (Semrock)
 - 594nm long pass emission filter (Semrock)
 - 482/18nm excitation filter (Semrock)
 - 2x Orca flash 4.0 sCMOS cameras (Hamamatsu)
- 2 LEX2 blue LED excitation source (Brainvision)
- 3 MSG10-1100S-SD Fibre optic light guide (Moritex)
- 4 ML-50 Condenser lens (Moritex)

Slicing

- 5 Tools for perfusion surgery: Rough forceps, Fine forceps, Scissors, Hemostat (Fine Science Tools)
- 6 27G needles, 10ml syringe, insulin syringe
- 7 Ketamine 100mg/ml and Xylazine 20mg/ml
- 8 Ice & ice box
- 9 Slicing ACSF (108 mM Choline Chloride, 3mM KCl, 26mM NaHCO₃, 1.25 mM NaH₂PO₄, 25 mM Glucose, 3 mM Sodium Pyruvate, 1mM MgCl and 2mM CaCl₂)

- 10 Bath ACSF (120 mM NaCl, 3 mM KCl, 23 mM NaHCO₃, 1.25 mM NaH₂PO₄, 10 mM Glucose, 1mM MgCl and 2mM CaCl₂)
- 11 VT1000S Vibratome (Leica)
- 12 250ml, 100ml and 1l beakers
- 13 Slice incubation chamber consisting of a 1l measuring cylinder with the base removed and replaced with fine netting such that it fits into the 1l beaker.
- 14 Cyanoacrylate glue
- 15 Filter paper
- 16 95% O₂ / 5% CO₂ Gas cylinder
- 17 Vapro 5520 Vapor Pressure Osmometer (Wescor)

Imaging Chamber

- 18 Minipuls 3 peristaltic pump (Gilson)
- 19 Tubing for delivery of ACSF and O₂/CO₂
- 20 Vibration damping table
- 21 SHD 42/15 Slice harp (Warner Instruments)
- 22 TC 324b Bath temperature controller (Warner Instruments)

Data Acquisition

- 1 Digidata 1322A Digitiser (Molecular Devices)
- 2 2x PCs with at least 32GB RAM to run the cameras. 1x PC capable of running pClamp software.
- 3 Matlab 2015a software including Image Acquisition and Image Processing packages (Mathworks)
- 4 pClamp Electrophysiology software (Molecular Devices)

Ephys

- 5 Electrode glass (World Precision Instruments)
- 6 PC-10 Electrode puller (Narishige)
- 7 Axopatch 200b patch clamp amplifier and CV 203BU headstage (Molecular Devices)
- 8 Isoflex Stimulus Isolator (A.M.P.I)
- 9 Master 8 (A.M.P.I)
- 10 MRE Micromanipulators and controller (Luigs and Neumann)

Misc

- 11 Bayonet Neill–Concelman (BNC) to BNC cables & SubMiniature version A (SMA) to BNC cables

3. Methods

The microscope images slices through a 10x/0.3NA water dipping objective and uses a Brainvision dichroic to split the emitted fluorescence into two channels corresponding to the fluorophores in the FRET pair of the dual differential emission GEVI (VSFP Butterfly 1.2 [13]). The emitted light is then focussed onto the active areas of two sCMOS cameras using inversely mounted 1x Planapo Leica objectives. The use of the Brainvision splitting optics leads to a very wide light path, reducing aperture losses relative to ‘dual view’ systems [14], [15] allowing large FOVs and a wide range of objectives to be accommodated. The microscope is mounted on a stage that can be moved relative to the slice chamber so that different locations within the brain slice can be imaged while stimulation electrodes are already placed into the slice. The microscope needs to be set up on a vibration isolation table with a frame and curtain that can block out ambient light that would contaminate the recordings. The table needs to be big enough to comfortably accommodate micromanipulators for electrodes and it is also useful to have a frame surrounding the air table but isolated from it which can be used for equipment such as peristaltic pumps for ACSF perfusion.

Each camera is controlled by a single computer; timing is controlled by a third computer that also records any electrophysiological traces using pClamp. For an acquisition the cameras are initialised by their respective computer and they wait for a trigger from pClamp which also controls the excitation shutter, stimulus isolator, and via a Master 8, a synchronisation LED and the peristaltic pump. Frame out pulses from the cameras, if available, can also be read into pClamp to ensure the timing of the images for post processing.

3.1 Setting up the rig

1. Connect the cameras to their computers and configure the drivers and control software. This can be commercially available software that comes with the cameras such as Hokawo (Hamamatsu), or custom written software in Matlab. It is essential that the software supports hardware triggering for the cameras. It is also useful at this stage to perform tests to ensure that the cameras are exactly synchronised frame-by-frame. This can be done by imaging an LED flashing at a fixed rate and ensuring that the light and dark frames appear at the same time points for both cameras.
2. Select the filters and dichroics required for the indicator to be imaged and install the filters into their mounts. Ensure that filters are clean, free of dust and mounted in the correct orientation.
3. Install the microscope optics into the z-stage on the air table above the slice chamber. The optics with the cameras mounted can be prone to toppling and so it

4. Connect the digitiser to the control computer. Using BNC cables connect the digitiser's digital control lines to the two camera's triggers using a splitter, to the excitation source shutter, the stimulus isolator and the Master 8 external in input triggering channel 1. Connect the Master 8 channel 1 to an LED on the air table and channel 2 to control the peristaltic pump. Internally connect channel 1 and 2 in the Master 8.
5. Configure the pClamp protocol so that the different outputs are triggered at the correct times. The cameras should be triggered first, then the Master 8, then the excitation source. The control flow is illustrated in figure 1. This can be checked again by imaging a fluorescent target and ensuring that the system operates as expected.
6. Connect the O₂/CO₂ gas tubing to the regulator so that the perfusion bath ACSF can be oxygenated before being pumped to the imaging chamber. Set up the peristaltic pump and perfusion system such that a flow rate of a minimum of 3ml/min of oxygenated ACSF is pumped through the imaging chamber. It is useful to use larger bore tubing on the outflow line so that flooding is avoided.
7. Attach the cameras to the optics and align them. To do this image a suitable flat target such as a black cross on a light background. First adjust the path lengths so that the two cameras are focussed in the same position. As an alignment tool it is useful to take an image of a flat target with both cameras and take the difference of the images. When displayed in false colour this will show misalignment clearly. Rotate one camera to fix rotation misalignments and use the dichroic adjusting knobs on the side of the dichroic housing to adjust translations. Iterate the process until satisfied.

3.2 Slice Experiments

Experiments with transgenic mice tend to involve older animals, and a detailed discussion of the challenges and techniques to overcome them can be found in reference [16]. It is important to ensure a good supply of well oxygenated ACSF when the slice is in the imaging chamber; however fluid movement can introduce artefacts into the image time courses and so it is useful to set up the peristaltic pump such that it transiently halts during acquisition of an image sweep. In our set up a Gilson Minipuls 3 Peristaltic Pump can be directly switched on and off via a hardware input and is controlled by pClamp via a Master 8. The stock bath and dissection ACSF prepared will keep at 4°C for three days; the required amount for the number of experiments planned for the week can be made up by scaling the quantities below.

3.2.1 Preparation – Day before Experiment

1. Prepare 1l of 2X bath ACSF and 1l of 1X dissection ACSF. Do not add any Mg or Ca as these can precipitate out of solution so it is best to add them just before use.

3.2.2 Preparation – Day of Experiment

1. Dilute 500ml of 2x bath ACSF to 1x with distilled H₂O to make 1l and measure out 200ml of dissection ACSF into a beaker.
2. Add 2ml and 1ml of 1M CaCl₂ and MgCl₂ respectively to the bath ACSF and 400µl and 200µl of the same to the dissection ACSF.
3. Measure and adjust the osmolality of both solutions to be 285 ± 5 mmol/Kg
4. Fill a large ice box with ice and place the dissection ACSF into the ice. Fill slice holding chamber with room temperature ACSF leaving ~300ml for perfusion through the imaging chamber.
5. Using 70% ethanol clean and prepare the area where the procedure is to take place, laying out all the equipment needed, clean the surgical tools and the vibratome chamber, blade and blade holder. Place the vibratome parts in a freezer at -20°C. Lay out the glue and filter paper.
6. Place cleaned tubing from the carbogen cylinder into the bath and dissection ACSF and bubble the solutions gently for at least 20 minutes prior to the procedure.

3.2.3 Slicing

1. Deeply anaesthetise the mouse expressing the indicator with a 100mg/kg 10mg/kg ketamine/xylazine IP injection [17]. Check that the mouse is anaesthetised using the pedal reflex.
2. Perfuse the mouse transcardially with 10–20ml of dissection ACSF. Once perfused, decapitate the mouse and remove the brain from the skull and place into ice cold dissection ACSF.
3. Remove the vibratome chamber from the freezer and place a line of superglue onto the mounting plate.
4. Take the brain out of the dissection ACSF and place on a piece of filter paper. Remove the cerebellum and, depending of area of interest cut a flat surface to glue to the vibratome plate using a razor blade wetted with ACSF.
5. Glue the brain anterior down on the vibratome plate, screw into the bath and fill the bath with dissection ACSF. Place the bath onto the vibratome and attach the vibratome blade.
6. If making coronal slices it is optional to hemisect the brain sagittally and remove the ventral white matter in two sections by making a diagonal cut below the cortex.
7. Make 300–350µm slices at around 0.6 mm/sec and 90Hz.
8. Transfer the slices from the vibratome bath to the recovery chamber using a 3ml Pasteur pipette with the end cut off.
9. Leave the slices for at least 2 hours to recover at room temperature.

3.2.3 Whilst Slices recovering

1. Wash through the imaging chamber and perfusion system with 50ml of each of 70% ethanol, distilled water and bath ACSF.
2. Ensure that the two camera channels are aligned.
3. Check that all control software is working and that there is adequate hard drive space.

3.2.4 Imaging & Electrophysiology

1. Using the pipette transfer the slice into the imaging chamber. Use a slice harp to anchor the slice. It is sometimes possible to physically rotate the chamber to ensure the slice is the same orientation to the camera.
2. Using a 1.6x objective take a transmitted/scattered light image and a fluorescence image for reference.
3. Change to a 10x objective and navigate to your section of interest. Take a scattering and fluorescence reference image again and then place your stimulating and/or field electrodes into your desired location.
4. Image the slice for a fixed duration with the same stimulus point so that trials can be averaged and compared. It is useful to stimulate approximately 1/3 of the way through the run to allow time for a baseline to be collected before the stimulus response and for the full response decay to be measured. We typically image in 3s trials, stimulating at the 1s point.
5. It is also useful to develop some quick analysis tools to look at ROIs after the run is complete to check for a response and get an idea its strength and the noise level.

4. Notes

There are a few key points that are essential for collecting useful datasets.

1

Choosing the correct filter set and excitation source for the indicator is critical and requires a compromise between collecting as many photons as possible whilst still rejecting as much light originating from sources other than the chosen indicator, such as autofluorescence and stray excitation light, as possible. Collection of photons is crucial for resolving the small changes in indicator brightness that arise from neuronal activity. Considering a shot-noise limited regime the signal-to-noise ratio, R , is given by

$$R \leq \frac{\Delta F}{F} \sqrt{n}$$

[3], where $\Delta F/F$ is the fractional fluorescence change of the indicator arising from the event to be detected and n is the number of collected photons. Typically not all photons collected

will have been emitted by the fluorophores that indicate the signal of interest; some photons collected at the camera will have originated from non-responsive indicator (e.g. indicator molecules not targeted to the plasma-membrane in the case of GEVIs), tissue autofluorescence, stray background light and, importantly, indicator-expressing cells that do not contribute to the signal of interest. If the fraction of photons coming from these sources is f_B , then the effective achievable R will be reduced to

$$R \leq \frac{\Delta F}{F} \sqrt{n} \sqrt{1 - f_B}$$

Careful selection of emission and excitation filters and labelling only cells that contribute to the signal of interest can minimise f_B . When using a FRET-based indicator such as VSFP Butterfly 1.2 there is likely less autofluorescence emitted around the redder fluorescent protein's wavelength as the excitation light being used is generally too short to effectively excite endogenous fluorophores with similar emission spectra (e.g. with a very long Stokes shift). This allows the use of a wider emission filter for this path to maximise collected signal photons. Conversely, as the bluer fluorescent protein's emission spectrum is more likely to overlap with that of fluorophores that will be excited by the excitation source and it is also more prone to direct leakage from the excitation source itself the filter on this channel must be more tightly limited around the peak of the indicator's emission spectrum. For each individual channel in the set up and indicator type this trade-off must be optimised to maximise R .

2

A second key point that will ease subsequent data analysis and interpretation is ensuring that the cameras in a dual colour acquisition set-up are aligned, and are collecting synchronous and regular image frames. If the two light paths are spatially misaligned then a suitable transform will have to be found to map the colour images onto each other before any dual colour analysis can be performed, which is an error prone and computationally expensive task. Non synchronous or irregular frame collection can distort the time courses of neural activity and confuse subsequent analysis. It is always best to use hardware based checks for timing issues. It is often possible to record the hardware frame out signal from the cameras and include it in the analysis as necessary. Another technique is to use an LED flash at the beginning and end of each acquisition to provide a timestamp for both cameras to compare their timing. To avoid timing issues in the first place it is important to use impedance matched connectors and splitters such as 50Ω BNCs when transmitting timing critical information such as camera triggers.

3

To remove any drift in baseline signal an exponential function can be fit to the fluorescence time course for each pixel, discounting the pixels showing a stimulation response (or alternatively use sweeps with no evoked responses). By dividing by this polynomial the time course is corrected for signal of interest-independent components due to chromophore bleaching, photoconversion, etc. and baseline-normalised, facilitating further analysis.

Depending on the noise level in the image it may be necessary to bin the image to achieve an adequate R. Depending on the magnification and NA of the objective used, this may have no effect on the resolution as the camera sensors used here are spatially oversampling.

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