Ex vivo Ca2+ 2PLSM measurements with genetically encoded probes

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We use this protocol and it's working

ABSTRACT

Ex-vivo slices obtained from mice that had previously undergone viral stereotaxic injections to express genetically encoded probes are used for fluorescence microscopy experiments. Because of the thickness of the slice tissue the preferred imaging method is the 2-photon excitation laser scanning microscopy (2PLSM).

A slice is placed on a small holding chamber on a temperature-controller motorized microscope stage and continuously bathed with a physiological solution, to which different drugs and pharmacological agents can be added in order to induce stimulatory events in the cells of interest or pharmacological manipulations, that will be detected as changed in
the fluorescence emitted by the probes. A variety of genetically-encoded Calcium (Ca2+) probes
exist, including probes for cytosolic Ca2+ (**GCaMP6**),mitochondrial Ca2+ (**mito-GCaMP6**), and ER Ca2+ (**G-CEPIA1er**).

GUIDELINES

Follow institutional guidelines and protocols.

MATERIALS

- 2PLSM optical workstation and computer with imaging softwares (see below)
- 488nm LED light source (or alternative light source to excite GFP)
- Artificial cerebro-spinal fluid (aCSF) (see below)

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- Holding chamber

- Blood-gas mixture (95% O2, 5% CO2) tank connected to bubblers.

- Slice holder

- Brain slices expressing genetically encoded Ca2+ probes “GECI” (e.g.: GCaMP6, mito-GCaMP6, G-CEPIA1-er) in holding chamber with aCSF (see protocols on stereotaxic surgeries for intracranial viral injections and ex vivo slice preparation)

- Peristaltic pump with tubing and connectors, including inlet and outlet to microscope’s imaging chamber

- Microscope heating system with probe

- Ca2+-free aCSF (see below)

- EGTA stock solution

- Ionomycin

- DMSO

- CaCl2 stock solution

- Waste solution collector

- 10% Ethanol in water (wash solution)

- 4% BSA (Bovine Serum Albumin) solution in water
(wash solution for ionomycin)

- Image analysis software (FIJI)

2PLSM optical workstation:

The laser scanning optical workstation embodies an Ultima dual-excitation-channel scan head (Bruker Nano Fluorescence Microscopy Unit). The foundation of the system is the Olympus BX-51WIF upright microscope with a LUMPFL 60X/1.0NA water-dipping objective lens. The automation of the XY stage motion, lens focus, and manipulator XYZ movement was provided by FM-380 shifting stage, axial focus module for Olympus scopes, and manipulators (Luigs & Neumann). Cell visualization and patching were made possible by a variable magnification changer, calibrated to
2x (100 µm FOV) as defined by the LSM bright-field transmission image, supporting a 1 Mpixel USB3.0 CMOS camera (DCC3240M; Thor Labs) with ~30% quantum efficiency around 770 nm. Olympus NIR-1 bandpass filter, 770 nm/100 nm, and microManager software were used with the patch camera. The electrical signals were sent and collected with a 700B patch clamp amplifier and MultiClamp Commander software with computer input and output signals were controlled by Prairie View 5.3-5.5 using a National Instruments PCI6713 output card and PCI6052e input card.

The 2P excitation (2PE) imaging source was a Chameleon Ultra1 series tunable wavelength (690-1040 mm, 80 MHz, ~250 fs at sample) Ti:sapphire laser system (Coherent Laser Group); the excitation wavelength was selected based on the probe being imaged (see below). Each imaging laser output is shared (equal power to both sides) between two optical workstations on a single anti-vibration table (TMC). Workstation laser power attenuation was achieved with two Pockels' cell electro-optic modulators (models M350-80-02-BK and M350-50-02-BK, Con Optics) controlled by Prairie View 5.3–5.5 software. The two modulators were aligned in series to provide enhanced modulation range for fine control of the excitation dose (0.1% steps over five decades), to limit the sample maximum power, and to serve as a rapid shutter during line scan or time series acquisitions.

The 2PE generated fluorescence emission was collected by non–de-scanned photomultiplier tubes (PMTs). Green channel (490–560 nm) signals were detected by a Hamamatsu H7422P-40 select GaAsP PMT. Red channel (580–630

nm) signals were detected by a Hamamatsu R3982 side on PMT. Dodt-tube-based transmission detector with Hamamatsu R3982 side on PMT (Bruker Nano Fluorescence) allowed cell visualization during laser scanning. Scanning signals were sent and received by the NI PCI-6110 analog-to-digital converter card in the system computer (Bruker Nano Fluorescence).

Solutions:

Different types of aCSF are adopted by different groups and are optimized for different for different preparations.

The procedure here described refers to experiments on substantia nigra pars compacta dopaminergic neurons.

The aCSF adopted for these experiments has the following composition: 135.75 mM NaCl, 2.5mM KCl, 1.25mM NaH2PO4, 25 mM NaHCO3, 2 mM CaCl2, 1 mM MgCl2, 3.5 mM glucose.

For Ca2+-free aCSF, CaCl2 is entirely substituted with MgCl2, for a total of 3mM MgCl2.

All aCSF solutions are constantly bubbled with 95% O2/5% CO2 blood gas mixture.

Ionomycin is prepared as a 10mM stock solution in DMSO.

10% Ethanol wash solution is prepared diluting Ethanol in water.

BSA wash solution is prepared as a 4% BSA dissolved in water.

Standard experimental procedure

1 Brain slices expressing GECI are obtained according to protocol and held at room temperature in

a chamber containing aCSF continuously bubbled with 95% O2/5% CO2 blood gas mixture until the moment of the experiment.

2 Turn on 2PLSM working station, including heated stage, and the computer.

3 Start running aCSF through the peristaltic pump, into the microscope chamber. Check also that the chamber outlet is removing solution from the chamber at the same rate, collecting it into a waste solution collector. A vacuum-based outlet is also recommended because this can help prevent overflow, if available.

4 Temperature probe should be inserted in the solution in the chamber. As the system is turned on, the temperature in the microscope chamber should reach 32-33C.

5 Turn on imaging software.

6 Once the temperature in the microscope chamber is approaching the desired one, transfer one slice from the holding chamber into the imaging chamber. Adjust its position accordingly and gently place a slice holder on top of it, making sure that it doesn't cover any region of interest.

7 With the eyepieces and using the LED as a light source, first verify with a low magnification objective the correct expression of the GECI and adjust the stage position so that the region of interest can be easily imaged. Then, with the 60X immersion objective find more specifically cells that could be good for imaging.

8 Once a good area has been identified, leave 60x objective immersed and in position, turn off LED and switch to the 2PLSM settings.

9 With the imaging software, preliminary adjust power and image acquisition settings (it is recommended starting from lower settings and increasing laser power and/or gain if needed) and start imaging in "live" mode. The 2P excitation wavelength adopted for the probes

mentioned above, all based on GFP, is 920nm. Other kinds of probe might yield better results with different excitation wavelengths.

10 Identify a cell/region to image from, optimize imaging settings including zoom, field of view, resolution, dwell time, frame rate. For experiments on somatic regions of substantia nigra dopaminergic neurons our preferred settings are: 256x256pixels image size, zoom 4, 12 us dwell time, restricting the region-of-interest so that the frame rate with these settings is 3-4 frames per second.

11 Laser power and PMTs gain are adjusted so that the fluorescence at baseline is bright but far from reaching saturation of the signal. In our conditions, signal saturation is experienced above 4095 fluorescence units; the baseline fluorescence for the object of interested is normally adjusted to average at around 1000 units. Background fluorescence in these conditions should be around 100 units. This should allow to easily measure fluorescence increases as well as decreases.

12 It is recommended to wait at least 10-15 mins after placing the slice in the chamber and lowering the 60x objective before starting any experiment. This should give sufficient time to the slice to stabilize and equilibrate properly with the working temperature of the chamber. Not waiting a sufficient time might result in changes in focus/movement and instable fluorescent baseline.

13 2PLSM imaging experiments with GECI can be performed either as time-lapse experiments or as continuous acquisitions, depending on the time-scale of the phenomenon under observation.

13.1 For slow pharmacological effects, time-lapse acquisitions are preferred. In this case, a series of frames are acquired at regular intervals. Each series of frames is then averaged and represents one time-point in the time lapse. Standard acquisition settings are 60 frames over ~15 sec, acquired every 10 minutes.

13.2 For faster effects (e.g. acute stimulation), continuous acquisitions are preferred. In PrairieView this is performed as a Brightness Over Time acquisition.

14 If desired, for a semi-quantitative approach, it is recommended to perform an in situ calibration of the dynamic range of each probe for each cell examined. This is obtained by estimating minimum and maximum fluorescence levels for each cell.

Calibration protocol

15 The calibration is performed in time lapse mode, with acquisitions every 10 minutes as

described above.

16 Several baseline (regular aCSF) acquisitions are collected, to make sure that the signal for the cell under examination is stable. Discard cells if no stable baseline can be established.

17 To obtain the minimum, a Ca2+-free aCSF is used, with addition of the Ca2+ chelator EGTA (0.5mM). The aCSF in the recording chamber is slowly substituted with Ca2+-free aCSF + EGTA, followed by Ca2+-ACSF + EGTA with addition of the Ca2+ ionophore ionomycin (1uM). The ionophore allows Ca2+ to cross biological membranes according to the concentration gradient, so in Ca2+ free ACSF this will lead to a depletion of Ca2+ from all cell compartments. Ionomycin might take a relatively long time to reach cells deeper in the tissue, and it is recommended to measure the minimum fluorescence over 30 minutes before moving to the maximum.

18 To obtain the maximum, ionomycin is added to regular aCSF. This is normally sufficient to strongly elevate cellular Ca2+ levels enough to saturate the high affinity probes. Adding 1mM CaCl2 for a total of 3mM CaCal2 is also recommended. Further increasing the concentration of Ca2+ shouldn't improve the maximum and has the potential downfall of precipitation of Ca2+ as Ca2+ phosphate, depending on the composition of the solution used for the experiments.

19 While acquisitions every 10 mins are appropriate for baseline and minimum measurements, when measuring the maximum it is recommended to increase the frequency of acquisitions (to 2-3mins), because the change will happen relatively fast and once the cells will be experiencing elevated Ca2+ levels they will start to deteriorate rapidly. For the same reason, it is recommended to measure the maximum after establishing the minimum, when ionomycin has had the time to fully incorporate into membranes. This should allow a fast change in the intracellular Ca2+ concentration instead of a slow leakage.

20 Because of rapid deterioration of cells in high Ca2+, it is preferred to perform the calibration protocol measuring minimum first followed by maximum.

21 Slices should be discarded after being treated with ionomycin.

22 It should be noted that calibration experiment on cytosolic Ca2+ (GCaMP6) have a higher yield compared to calibration experiments on Ca2+ in subcellular organelles (mito-GCaMP6, G-CEPIA1er), because these organelles can become damaged during the different steps, which can

prevent the estimation of the maximum fluorescence.

After the experiment

23 Discard slices and waste solutions according to institutional protocols.

24 Carefully wash tubing, microscope chamber, slice holder, 60X objective and any part that comes in contact with the experimental solution. Water followed by 10% Ethanol is generally recommended. After using solutions containing ionomycin it is recommended to use also a 4% solution of BSA in water, because this will help absorbing residual ionomycin and prevents contamination.

25 Turn off all the equipment according to instructions.

26 Export data and proceed with image analysis.