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Ex vivo ATP/ADP measurements

Cecilia Tubert[1]

[1]Northwestern University, Feinberg School of Medicine

Surmeier Lab

Cecilia Tubert

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 Perceval probe.

ATTACHMENTS

Ex vivo ATP_ADP measurements.docx

GUIDELINES

Follow institutional guidelines and protocols.

MATERIALS

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)

- Holding chamber

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

- Slice holder

- Brain slices expressing genetically encodedATP/ADP probe PercevalHR in holding chamber with aCSF

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

- Heating system with probe

- Oligomycin

- 2-deoxyglucose (2-DG)

- DMSO

- 2-DG aCSF

- Waste solution collector

- 10% Ethanol in water (wash solution)

- Image analysis software

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 NaH_2PO_4, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 3.5 mM glucose.

For 2-DG aCSF, glucose is entirely substituted with 2-deoxyglucose (2-DG, 3.5mM).

All aCSF solutions are constantly bubbled with 95% O_2/5% CO_2 blood gas mixture.

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

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

Procedure:

1 - Brain slices expressing PercevalHR are obtained according to protocol and held at room temperature in a chamber containing aCSF continuously bubbled with 95% O_2/5% CO_2 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 PercevalHR 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. PercevalHR is a dual-excitation wavelengths probe: the 2P excitation wavelengths recommended for these experiments are 820nm (ADP-sensitive) and 950nm (ATP-sensitive), but this might be optimized depending on different imaging systems. Either wavelength can be used to adjust focus/position of the cell(s) under investigation, but the settings for both wavelengths will require separate adjustments before starting the actual experiment.

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.

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, 12us dwell time, restricting the region-of-interest so that the frame rate with these settings is 3-4

frame per second.

11   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.

12   PercevalHR experiments are normally performed with dual excitation at 950nm and 820nm, in rapid sequence. The ratio values obtained from individual dual acquisitions are collected as a time lapse. In our setup, this is normally achieved by creating a time-series acquisition protocol that switch the 2P laser wavelengths in sequence, automatically change the power/gain settings to the set values for each wavelength and allow for some time (10s) for the light path to stabilize before the acquisition.

13   Laser power and PMTs gain are adjusted and saved for each individual wavelength. In general, the goal is for the baseline fluorescence to be reasonably 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 100-200 units (normally background is slightly higher for 820nm). This should allow to easily measure fluorescence increases as well as decreases, for both wavelengths. It is also recommended to adjust the settings so that the baseline ratio between the 950/820nm fluorescence measured in the region of interest is close to 1, because this will make easier to monitor the progress of the experiment and subsequent analysis.

14   Time-series experiments are normally performed by collecting dual-wavelength acquisitions at intervals of 5 minutes. For each acquisition, normally 5 frames are acquired per wavelength, and averaged during analysis.

15   It is recommended to take several control acquisitions to verify that baseline is stable. The 950/820 ratio as well as the individual fluorescence values measured for 950 and 820 excitations should be stable. Discard cells with unstable baselines.

16   Time-series experiments can be performed to test the effect of different treatment or stimuli on the ATP/ADP ratio in a cell.

17   A common protocol to estimate the relative contributions of mitochondrial oxidative

phosphporylation and glycolysis to the cell's bioenergetic status is based on the bath application of the mitochondrial complex V (ATP synthase) inhibitor oligomycin (10uM) followed by inhibition of glycolysis by substituting the glucose in the aCSF with a non-hydrolizable analogue 2-deoxyglucose (2-DG).

18   Oligomycin (10uM) is bath applied in standard aCSF. As it can take some time for the drug to reach its target and to achieve its full effect, it is recommended taking several PercevalHR acquisitions at regular intervals (5 mins) until the ratio reaches a plateau. This normally takes about 30 mins (6 acquisitions).

19   The experiments is ended by inhibiting glycolysis. The perfusion solution is switched from regular aCSF to 2-DG aCSF always in presence of oligomycin. The plateau ratio value obtained in 2-DG aCSF + oligomycin represent the minimum PercevalHR ratio for the cell/experiement. The effect of 2-DG substitution is relatively fast (10-20mins).

20   Normally, the fluorescence levels for 950 and 820 excitations move in opposite directions during metabolic manipulations.

21   It should be noted that during metabolic manipulation the morphology of the cell under analysis or of the surrounding tissue might undergo some changes, which can affect the focus and the raw fluorescence values detected. This is usually particularly evident in 2-DG aCSF + oligomycin. Calculating the 950/820 ratio will compensate these phenomena, but it is recommended to properly adjust focus/position between acquisitions.

22   2PLSM imaging of PercevalHR can be combined with electrophysiology, as time-lapse acquisition while monitoring the firing of the cell under investigation. For experiments requiring high time resolution single-wavelength (950nm) continuous brightness-over-time acquisitions can be synchronized with voltage recordings.

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.

25 Turn off all the equipment according to instructions.

26 Export data and proceed with image analysis.