



OCT 14, 2023

OPEN ACCESS



DOI:
dx.doi.org/10.17504/protocols.io.j8nlkomb1v5r/v1

Protocol Citation: Cecilia Tubert 2023. Ex vivo roGFP measurements. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.j8nlkomb1v5r/v1>

MANUSCRIPT CITATION:

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Protocol status: Working
 We use this protocol and it's working

Created: Oct 14, 2023

Ex vivo roGFP measurements

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ABSTRACT

This protocol details the steps to obtain ro-GFP measurements of ex vivo brain slices in a 2PLSM.

ATTACHMENTS

[roGFP 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 encoded redox probe (roGFP, mito-roGFP,

Last Modified: Oct 14, 2023

other targeted roGFPs) in holding chamber with aCSF

PROTOCOL integer ID:
89297

- Peristaltic pump with tubing and connectors, including inlet and outlet to microscope's imaging chamber
- Heating system with probe
- Dithiothreitol
- Aldrithiol
- Ethanol
- 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 μ m 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 nm, 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, constantly bubbled with 95% O₂/5% CO₂ blood gas mixture.

Dithiothreitol should be freshly dissolved in water at a 1M stock concentration before the experiment.

Aldrithiol stock solution is prepared in 100% Ethanol at a concentration of 50 mM.

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

Procedure:

- 1 Brain slices expressing roGFP probes 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 32-33°C.
- 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 roGFP 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. The recommended 2P excitation wavelength for roGFP is 920nm.
- 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 Laser power and PMT's gain are adjusted to optimize experimental measurements. 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. This should allow to easily measure fluorescence increases as well as decreases.
- 13 Time-series experiments are normally performed by collecting acquisitions at intervals of 10 minutes. For each acquisition, normally 60 frames are acquired.
- 14 It is recommended to take several control acquisitions to verify that baseline is stable. Discard cells with unstable baselines.

- 15 Time-series experiments can be performed to estimated baseline oxidation or to test the effect of different treatment or stimuli.
- 16 The standard protocol to estimate the relative oxidation is based on the application of a reagent that gives full reduction of the probe Dithiothreitol (DTT, 2 mM) followed by a strong oxidizing agent Aldrithiol (Ald, 100 microM).
- 17 DTT (2 mM) is bath applied in standard aCSF. The reducing agent should lead to an increase in the roGFP fluorescence. As it can take some time for the drug to reach its target and to achieve its full effect, so it is recommended taking several roGFP acquisitions at regular intervals (10 mins) until the fluorescence reaches a plateau.
- 18 Following DTT reaches its plateau fluorescence, Ald (100 microM) is bath applied. The oxidation of the probe should lead to a decrease in its fluorescence. Collect several acquisitions (10 mins apart) until the fluorescence stabilizes.

After the experiment:

- 19 Discard slices and waste solutions according to institutional protocols.
- 20 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.
- 21 Turn off all the equipment according to instructions.
- 22 Export data and proceed with image analysis.

- 23** The relative oxidation is calculated as a percentage of the full range of the probe oxidation, with the maximum fluorescence obtained with DTT set as minimum oxidation, and the minimum fluorescence measured in Ald as full oxidation.