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

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Protocol for Neuronal Live-imaging of primary cultures

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ABSTRACT

Neurons stand out as remarkably intricate cells. The development of live-imaging techniques on primary neurons expressing genetically engineered biosensor has proven pivotal in advancing our comprehension of neuronal function at both cellular and molecular level. This protocol introduces a concise method for live-imaging neuronal activity.

ATTACHMENTS

Protocol for neuronal Live-Imaging_RyanLab.pdf

GUIDELINES

The animals should be used in accordance with protocols approved by national and institutional regulatory organizations.

MATERIALS

Media Supplies

- 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Alomone Labs, Cat. No. C-141)
- Calcium Chloride (Millipore-Sigma, Cat. No. C3881)
- DL-2-Amino-5-phosphonovaleric acid (APV; Millipore-Sigma A5282)
- Glucose (Millipore-Sigma, Cat. No. G7021)
- HEPES (Millipore-Sigma, Cat. No. H3375)
- Magnesium Chloride (Millipore-Sigma, Cat. No. M0250)
- Potassium Chloride (Millipore-Sigma, Cat. No. P9333)
- Sodium Chloride (Millipore-Sigma, Cat. No. S5761)

Other Supplies

- Ethanol (200 proof; Koptec, Cat. No. 6175)

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- Grease (Millipore-Sigma, Cat. No. Z273554-1EA)
- Wipes (Kimtech Science)
- P35 dishes (Falcon, Cat. No. 351008)

Stock solutions

APV (25mM Stock)

1. Add 20L ddH_2O to whole bottle of APV
2. Sonicate at ~55C for 30 minutes.
3. Make 1mL aliquots and store at -20C.

CNQX (10mM Stock)

1. Add 10 mL of ultrapure H_2O to the whole bottle of CNQX.
2. Vortex until dissolved.
3. Make 1 mL aliquots and store at -20 ^{O}C.

Media solution

Imaging Solution (119mM NaCl, 2.5mM KCl, 5mM D-Glucose, 50mM HEPES, 2mM $CaCl_2$, 2mM $MgCl_2$, 50uM APV, 10uM CNQX)

1. Add 450mL of ddH_2O
2. Add 3.47g of NaCl
3. Add 0.45g of Glucose
4. Add 5.96g of HEPES
5. Add 1.25mL of KCl (1mM stock prepared in ddH_2O)
6. Add 500uL of $CaCl_2$ (2mM stock prepare in ddH_2O)
7. Add 500uL of $MgCl_2$ (2mM stock prepare in ddH_2O)
8. Add 1mL of APV (25mM stock)
9. Add 500uL of CNQX (10mM stock)
10. Adjust pH to ~7.4 with NaOH
11. Add water to 500mL
12. Confirm pH is correct
13. Filter to a 500mL bottle

Equipment

- 40x, 1.3 N.A Objective (like Zeiss-Fluar)
- 488nm Laser (like Coherent-OBIS 488nm LX Laser)
- CO_2 Incubator (like VWR Symphony)
- EMCCD Camera (like Andor iXon Ultra 897)
- Epifluorescence Microscope (like Zeiss ObserverA1)
- Filter cube set for GFP excitation and emission (like Chroma filters)
- Laser speckle reducer (like Optotune LSR 3005)
- Laser/stage/stimulation-Master board controller (like a custom-designed Arduino

board)

- Light transmission microscope (like Olympus CK40)
- Mercury arc lamp (like Zeiss HBO 100)
- Perfusion system (like Automate Scientific Valvelink 8.2)
- Microscope XYZ Stage (like Applied Scientific Instrumentation stage)
- Stimulus isolator (like World precision Instruments A385)
- Temperature controller for Objective (like Minco)

SAFETY WARNINGS

- Laser light, because of its special properties, can cause safety hazards not related to light from common sources. The safe use of lasers requires that all laser users and all persons near the laser system understand the possible danger. The safe use of the laser depends on the user understanding the instrument and the properties of coherent, strong beams of light.
- Direct eye contact with the output beam from the laser will cause damage and possible injury to the eyes.

BEFORE START INSTRUCTIONS

- Turn on live-imaging setup: Lasers, microscope, mercury arc lamp, stimulus isolator, XYZ stage, imaging computer.
- Open acquisition and controller programs (i.e. camera program, laser's controller, ImageJ).
- Make sure the EMCCD camera has reach the working temperature (~ -80°C) before start imaging.

Mounting the coverslip to recording chamber

3m

1 Add 2 mL of 'Imaging Solution' into a P35 dish.

2 Cut a piece of a new coverslip and fix it with some grease into the 'Stimulator insert' (Figure 1).

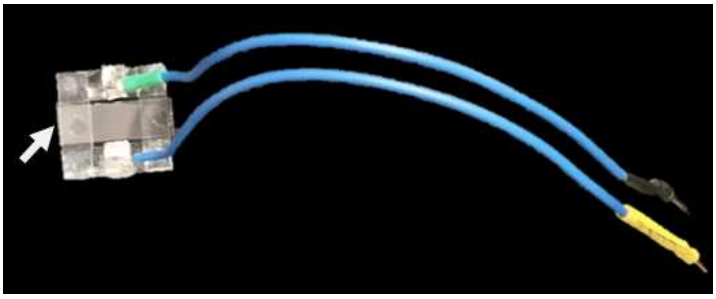

Figure 1. Stimulator Insert with a coverslip piece (white arrow)

3 Add a drop of grease to every corner (bottom face) of the ‘Stimulator insert’ (Figure 2).

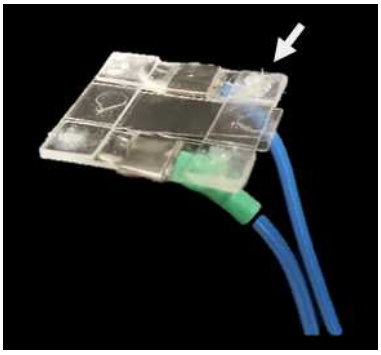

Figure 2. Stimulator Insert: Bottom face corners with a drop of grease (white arrow).

4 Add grease around the circle (facing up) of the ‘platform chamber’ (Figure 3).

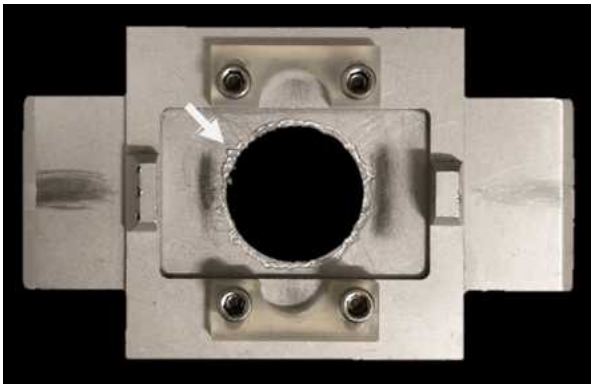

Figure 3. Platform chamber with grease (white arrow).

5 Take a DIV14 – DIV18 neuronal cultured dish from the incubator.

6 Assess for cell density, health and overall conditions using a basic light transmission microscope to

ensure the suitability of the dish before starting the experiment.

7 Place the coverslip containing platted cells into the petri dish prepared at go to step #1, and leav 30s for 00:00:10 to 00:00:20 to equilibrate with the 'Imaging Solution'.

8 Attach the coverslip to the 'Stimulator insert'; with the help of some tweezers make sure every corner of the 'Stimulator insert' is well attached to the coverslip by the grease.

9 Seal the 'Stimulator insert' to the 'platform chamber', clean any excess of grease that might be around and make sure there is no leak.

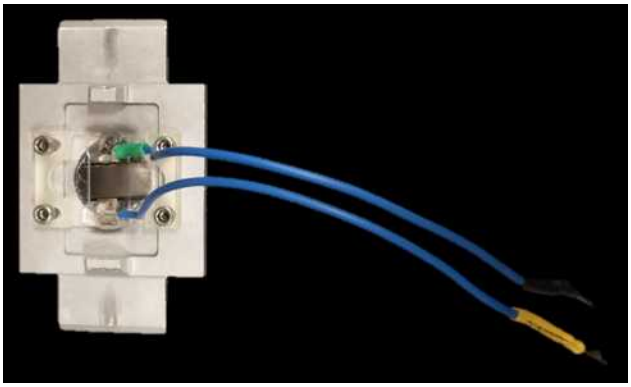

Figure 4. Recording Chamber assembled.

10 Clean the bottom of the coverslip with a wipe with Sample, making it ready for imaging.

Checking for transfected neurons under the microscope

5m

11 Place the 'recording chamber' into the microscope's stage.

12 If electrically stimulating neurons, connect the 'recording chamber' to the stimulus isolator.

13 Start prefunding 'Imaging Solution' trough the coverslip at a speed of ~ 100 uL min-1 . Make sure all perfusion lines with additional test solutions are calibrated to run at the same speed.

14 Using transmission light find the focal plane where neurons are located.

15 Change microscope illumination to the mercury arc lamp, making sure the desire dichroic filter is in place. (i.e. GFP-filter cube).

16 Verify the presence of transfected neurons based on their baseline fluorescence emission. If the biosensor's baseline fluorescence is insufficiently bright due to its inherent nature (i.e. vGLUT-pH), add a suitable solution (i.e. NH_4Cl-Tyrode's) into the chamber that may enhance the biosensor signal, facilitating the identification of transfected neurons.

17 Save all localizations where transfected neurons are found.

18 Proceed with the imaging protocol.

Neuronal Imaging Protocol

19 Flip the microscope mirror from the mercury arc lamp towards the laser path.

20 Make sure the following programs are open at the 'Imaging-setup computer': EMCCD camera control software (like Andor-Solis program), laser control software (like Coherent connection), master control program (like a custom-designed Arduino controller program) and image analysis program (like ImageJ

with Time-Analyzer plugin).

21 Load the desired acquisition settings protocol for imaging in the EMCCD camera control software.

22 Select one of the previously saved stage localizations, re-focus and move the neuron/boutons to a desire field of view, ready to be imaged.

23 If electrically stimulating, make sure that the correct settings are selected (i.e. number of pulses, stimulation frequency, time to first pulse) and ready to Go when triggered by camera action.

24 When ready for imaging, proceed to record.

Additional notes

25 This live-imaging protocol can be adapted to be used with ratiometric biosensors (i.e. iATPSnFR2-HALO), by using the appropriate dual filter cube corresponding to the different fluorescent emissions and, by switching lasers between frames when image acquisition is performed.