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We use this protocol and it's working

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Hippocampal Cultures, lentivirus production, vGLUT1-pHluorin imaging and analysis

Leonardo A Parra-Rivas[1]

[1]University of California, San Diego

Subhojit Roy Lab

Leonardo A Parra-Rivas
University of California, San Diego

ABSTRACT

Hippocampal Cultures, lentivirus production, vGLUT1-pHluorin imaging and analysis

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Hippocampal Cultures

1 Before dissection, add 100 µL of 1 mg/mL Poly-d-Lysine solution on to the inner well of each Mattek dish. Spread evenly and allow the coating to proceed for 2 h at room temperature. After 2 h wash the dishes thrice with ddH_2O. Dry the dishes thoroughly using an aspirator and let them sit in the hood until neurons are ready to be plated.

2 Dissect out the hippocampus from the P0–P1 mice pups after rapid decapitation using a standard dissection protocol. A good tutorial video demonstrating the procedure for hippocampal neuronal culture is available on the following website: http://www.ejnblog.org/2011/11/04/preparation-of-neuronal-cell-cultures/. Place the dissected hippocampi in ice-cold HBSS buffer before enzymatic treatment. Two hippocampi from each brain yield approximately 60,000 cells after dissociation. Around 25,000 cells are plated per dish, so the number of hippocampi to be dissected needs to be calculated according to the number of dishes being plated. All dissections are carried out in ice-cold HBSS buffer.

3 Once the desired number of hippocampi have been collected in ice-cold HBSS transfer them to 10 mL of pre-warmed 0.25 % Trypsin–EDTA at 37 °C. Incubate the hippocampi for 15 min at 37 °C in a water bath. After 15 min, stop enzymatic digestion by transferring the hippocampi to 10 mL blocking buffer.

4 Transfer the hippocampi from the blocking solution to 5 mL of 1× PBS. Carry out two more washes in 5 mL 1× PBS. Transfer the hippocampi to a vial containing 1 mL of plating media for dissociation.

5 Dissociate hippocampi in 1 mL plating media by triturating 5–8 times with an unused P1000 pipette tip. The plating media should turn slightly turbid during the dissociation process. Once this is achieved and no large chunks of tissue are seen floating in the media, the cell suspension is passed through a 70 µm filter. 10 µL of the sterile cell

suspension is then used to count cells on a standard hemocytometer.

6 After counting, about 25,000 cells/100 µL of the cell suspension are plated on to the center well of each poly-d-lysine coated dish. Adjust the cell dilution close to this value before plating.

7 After plating, allow the cells to recover for 1 h in an incubator (5 % CO_2, 37 °C). Then add 1.5 mL of neurobasal/B27 (NB/B27) media to the culture dish and place the dish back in the CO_2.For synaptic targeting experiments, neurons were transiently transfected on DIV-13 using Lipofectamine 2000 (Invitrogen) and imaged at DIV 17-21.

lentivirus production

8 HEK293T cells (RRID:CVCL_0063) were maintained in DMEM+Glutamax supplemented with 10% FBS and 1% penicillin- streptomycin for lentivirus production. Before transfection, $6x10^6$ cells were plated in 15cm dishes (Genessee Scientific) for 14-16 hrs.

9 HEK cells were transfected with the targeting plasmid and two helper plasmids (psPAX2 (*RRID:Addgene_12260)* and pMD2.G (*RRID:Addgene_12259)*) at a 2:1.5:1 molar ratio, using ProFection (Promega).

10 The supernatant was collected and concentrated using LentiX (Takara) two to three days later. The viral pellet was resuspended in 1/100th of the supernatant volume of sterile HBSS and stored at −80°C.

11 For viral transduction, lentiviruses were added to each well of neurons at DIV 3 at MOI=5. In all cases, almost 100% transduction was achieved with the lentiviruses, as confirmed by immunostaining.

vGLUT1-pHluorin imaging and analysis

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For the lentiviral vGLUT1:pHluorin experiments, cultured neurons were plated at 60,000 cells/cm^2 density. DIV-3 neurons were infected with lentiviruses carrying α-syn tagged at the C-terminus to mScarlet, or mScarlet alone (multiplicity of infection or MOI=2.5).

13 Subsequently, lentiviruses carrying vGLUT1:pHluorin (MOI=2.5) were added to DIV-5, and the transduced neurons were cultured to maturity (DIV17-DIV21) before imaging. Near 100% infection efficiency of the mScarlet-tagged constructs was confirmed before using the coverslip for pHluorin experiments.

14 For transfected vGLUT1:pHluorin assays, P0-P1 neurons were electroporated with the respective constructs using an Amaxa 4D-Nucleofector™ System (Lonza Inc., Walkersville, MD, USA) with the P3 Primary Cell 4D-Nucleofector X Kit S (V4XP-3032) and program CL-133. The volume of the cell suspension was 20 µl per reaction, and the cell density ranged from 1×10^7 to 1.5×10^7 cells/ml.

15 Cells were plated at a 60,000 cells/cm^2 density onto poly D-lysine-coated coverslips after electroporation and cultured to maturity (DIV14-DIV17) before imaging. Neurons were imaged live using an inverted motorized epifluorescence microscope (Olympus,IX81) fitted with a Prime 95B camera. The coverslips were mounted into a Chamlide EC magnetic chamber
(Live cell Instrument, ON, Canada) in Tyrode solution (pH 7.4) containing (in mM):
119 NaCl, 2.5 KCl, 2 CaCl2, 2 MgCl2, 25 HEPES, 30 glucose, 10 µM 6-cyano-7-nitroquinoxaline-2,3-dione 015 (CNQX, TOCRIS bioscience #0190), and 50 µM D,L-2-amino-5-phosphonovaleric acid (AP5, TOCRIS bioscience #0105).NH4Cl perfusions were done with 50mM NH4Cl in substitution of 50 mM NaCl (pH 7.4).

16 For field stimulation, 10 V/cm pulses were applied at 10 Hz for 60 seconds using a Model 4100 Isolated High-Power Stimulator (A-M systems, Sequim, WA). Incident excitation (Lumencor LED, Spectra X) was attenuated 10-fold, and images were acquired with 500 ms exposures at three-second intervals for three minutes.

17 For analysis, regions of interest (ROIs) were placed on each bouton, and average intensities were obtained for each frame within the time-lapse. Fmax was defined as the maximal fluorescence after NH4Cl perfusion. Baseline F0 was defined as the average fluorescence of the initial 10 frames before stimulation [(F0 = average (F1:F10)]. Fluorescence intensity of a bouton at a given time point (F) was normalized to F0 and Fmax and expressed as (F-F0) / (Fmax-F0).