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

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Live Imaging of Primary Mouse Neuron Cultures

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ABSTRACT

This protocol describes live imaging of primary neuron cultures. Included are methods for preparing hippocampal or dopamine neuronal cultures from neonatal mouse brain tissue. The imaging described involves labeling of dopamine neurons with a fluorescent DAT ligand and using virally encoded pHlourin sensors to measure vesicular release of neurotransmitter as the neurons are electrically stimulated. This technique was used in Jain et al., 2023 to compare vesicular release in neurons between various transgenic knockout mouse lines.

PROTOCOL MATERIALS

Basal Medium Eagle (BME) **Thermo Fisher Catalog #21010046** — Step 20

GDNF Recombinant Human Protein **Thermo Fisher Catalog #PHC7045** — Step 20

HyClone Fetal Bovine Serum **Fisher Scientific Catalog #SH3039603** — In 2 steps

D-( )-Glucose solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G8769** — Step 8

B27 supplement without retinoic acid (50x) **Gibco, ThermoFisher Catalog #17504044** — In 3 steps

Neurobasal™ Medium **Gibco - Thermo Fisher Catalog #21103049** — In 2 steps

Glutamax (100x) **Gibco - Thermo Fischer Catalog #35050-061** — In 2 steps

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Preparation of Hippocampal Cultures

1 Coat coverslips with Poly-L-lysine

2 Collect mouse pups at postnatal day 0

2.1 For Jain et al., 2023, cultures were prepared from WT, β3A KO, β3B KO, and *mocha* mice

3 Decapitate and remove brain, dissect out hippocampi from each hemisphere into Hank's balanced salt solution (HBSS) containing 10 mM HEPES and 20 mM glucose

4 Digest with papain (20 units/mL) in papain digestion buffer (HBSS with 20 mM $CaCl_2$, 5 mM EDTA, 0.2 mg/mL L-Cysteine, 10 mM HEPES at pH 7.4) for 15 minutes

5 Wash hippocampi three times with HBSS containing 10 mM HEPES and 20 mM glucose

6 Triturate hippocampi to get a single cell suspension

7 Using a hemocytometer, count the density of cells in the suspension

8 Plate cells onto the poly-L-lysine-coated coverslip at a density of 350 cells/mm^2 in Minimal Essential Medium containing:

- 1X B27 supplement
  B27 supplement without retinoic acid (50x) **Gibco, ThermoFisher Catalog #17504044**
- 5% Fetal Bovine Serum (FBS)
  HyClone Fetal Bovine Serum **Fisher Scientific Catalog #SH3039603**
- 21 mM glucose D-(+)-Glucose solution **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G8769**

9 Store cultures in incubator at 37 °C

10 After day 1 *in vitro* (DIV1), replace 3/4 of the medium with Neurobasal medium Neurobasal™ Medium **Gibco - Thermo Fisher Catalog #21103049** containing:

- 1X B27 supplement
  B27 supplement without retinoic acid (50x) **Gibco, ThermoFisher Catalog #17504044**
- GlutaMAX Glutamax (100x) **Gibco - Thermo Fischer Catalog #35050-061**

11 Infect cells at DIV4-5 using lentiviral vectors

11.1 For Jain et al., 2023, viruses encoding VGLUT2-pH, VMAT2-pH, or VGAT-pH were used

12 At DIV 6-7, inhibit glial proliferation by treating cultures with 4 µM cytosine arabinoside (Ara-C)

Preparation of Dopamine Neuron Cultures

13 Coat coverslips with poly-L-lysine and laminin; plate astrocyte monolayer onto coverslips

14 Collect mouse pups at postnatal day 0

15 Decapitate and remove brain, dissect out midbrain (including ventral tegmental area and substantia nigra) from each hemisphere into Hank's balanced salt solution (HBSS) containing 10 mM HEPES and 20 mM glucose

16 Digest with papain (20 units/mL) in papain digestion buffer (HBSS with 20 mM $CaCl_2$, 5 mM EDTA, 0.2 mg/mL L-Cysteine, 10 mM HEPES at pH 7.4) for 15 minutes

17 Wash digested tissue three times with HBSS containing 10 mM HEPES and 20 mM glucose

18 Triturate tissue to get a single cell suspension

19 Using a hemocytometer, count the density of cells in the suspension

20 Plate cells onto the pre-prepared coverslips at a density of 1000 cells/mm^2 in medium consisting of 60% Neurobasal Medium Neurobasal™ Medium **Gibco - Thermo Fisher Catalog #21103049**, 30 % Basal Eagle Medium Basal Medium Eagle (BME) **Thermo Fisher Catalog #21010046**, 10% FBS HyClone Fetal Bovine Serum **Fisher Scientific Catalog #SH3039603** containing:

- 1X B27 supplement
  B27 supplement without retinoic acid (50x) **Gibco, ThermoFisher Catalog #17504044**
- 2 mM GlutaMAX Glutamax (100x) **Gibco - Thermo Fischer Catalog #35050-061**
- 10 ng/mL glial cell line-derived neurotrophic factor (GDNF)
  GDNF Recombinant Human Protein **Thermo Fisher Catalog #PHC7045**
- 1X penicillin/streptomycin

21 At DIV 2-4, infect with lentivirus

21.1 For Jain et al., 2023, cells were infected with lentivirus encoding VMAT2-pH or VGLUT2-pH

Live Imaging

22 Maintain cultures until ready for imaging. Live imaging of cultures is performed at DIV14-16 for hippocampal cultures and DIV13-15 for dopamine neuron cultures

23 Prepare Tyrode's buffer:

- 119 mM NaCl
- 25 mM HEPES
- 2 mM $CaCl_2$
- 2 mM $MgCl_2$
- 2.5 mM KCl
- 30 mM glucose

Adjust pH to 7.4

24 For midbrain cultures, label dopamine neurons, incubate dopamine neuron cultures in Tyrode's buffer (119 mM NaCl, 25 mM HEPES, 2 mM $CaCl_2$, 2 mM MgCl2, 2.5 mM KCl, and 30 mM

24.1 Make Tyrode's +JHC1-64 by adding the fluorescent DAT ligand JHC1-64 at 30 nM to Tyrode's buffer

24.2 Incubate dopamine neuron culture in Tyrode's +JHC1-64 for 5 minutes at Room temperature

24.3 Rinse neurons in Tyrode's buffer (no JHC 1-64)

25 Add fresh Tyrode's buffer to culture dishes, mount coverslips in a perfusion and stimulation chamber of a TE300 inverted epifluorescence microscope

26 Monitor fluorescence:

- For pHlourin, use 470/40 nm excitation and 525/70 nm emission bandpass filters
- For JHC1-64, use 545/25 nm excitation and 605/70 nm emission bandpass filters

Use the red channel to identify dopamine neurons then switch to the pHlourin channel for experiments

27 Place platinum-iridium stimulating electrodes near the dopamine neuron being observed

28 Acquire images, typically at 1 Hz except for Fluo-5F imagine where images are acquired at 10 Hz

29 While acquiring images, evoke action potentials using 1 ms bipolar current pulses through the stimulating electrode at 5-10 V/cm