Culture of Mouse Giant Central Nervous System Synapses and Application for Imaging and Electrophysiological Analyses

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Abstract

Primary neuronal cell culture preparations are widely used to investigate synaptic functions. This chapter describes a detailed protocol for the preparation of a neuronal cell culture in which giant calyx-type synaptic terminals are formed. This chapter also presents detailed protocols for utilizing the main technical advantages provided by such a preparation, namely labeling and imaging of synaptic organelles, and electrophysiological recordings directly from presynaptic terminals.

Keywords

Giant synapse, neuronal cell culture, neurotrophin-3, calyx of Held, synaptic vesicles, capacitance measurements, synapse imaging

1. Introduction

Primary dissociated culture and acute brain slice preparations are widely used for neuronal synaptic studies. In acute brain slices, giant mammalian nerve terminals are large enough to allow whole-cell patch clamp access for electrophysiological recording and molecular loading. Alternatively, culture systems have monolayer cell organization simplifying imaging experiments, and furthermore culture longevity of about one month allows studies with genetic or environmental manipulations. Studies in acute brain slices of a giant mammalian synapse, the calyx of Held, have revealed major mechanisms of neurotransmission and neuromodulation ***(1-4)***. Genetic manipulations and imaging in cultured synapse preparations have revealed functional aspects of synaptic proteins ***(5-8)*** and mechanisms underlying synaptogenesis ***(9, 10)***. To combine advantages of these two types of preparations, we have developed a primary culture of calyx-type giant synapse using co-culture of dissociated mouse brainstem neurons from the cochlear nucleus (CN) and medial nucleus of the trapezoid body (MNTB) ***(11)***. In addition to its utility for genetic manipulations and imaging assays such as pHluorin (***12***) and styryl dyes (FM dye) ***(13)*** methods, this preparation allows simultaneous imaging and electrophysiological recordings from the same presynaptic terminals. Such opto-electrical readouts will facilitate understanding of synaptic mechanisms by bridging molecular and electrophysiological findings.

For labeling synaptic vesicles, fluorescent-conjugated antibodies directed against the luminal domain of synaptotagmin and fluorescent nanoparticles have been used in cultured mammalian synapses ***(14-16)***. Using this cultured giant synapse preparation, we

have recently developed labelling and tracking methods for synaptic vesicles to analyze their dynamic properties ***(17)***.

Neurotrophins are involved in synaptic function and modulation ***(18)***. In this culture, neurotrophin-3 (NT-3) is required for the formation and survival of the calyx-type giant synapses. Neutralization of NT-3 with its specific antibody in the culture strongly suppresses calyceal synapse formation and structural maturation ***(11)***. Thus, in addition to the above technical advantages, this preparation can be used to investigate the effect of neurotrophic factors on synaptic formation, maturation, and functions. In the following chapter, we describe details for this culture preparation and further provide protocols for imaging and electrophysiological techniques applicable to this giant synaptic terminal.

2. Materials

2.1. Equipment and labware

1. Sterile 35 mm dishes with thin plastic culture surface and low walls to allow patch/stimulating electrode access. The hydrophobic µ-Dish $^{35mm,\ low}$ from Ibidi (#80131) gives the best results in our experience..
2. Confocal laser scanning microscope (e.g. Zeiss LSM780) or Spinning-disc confocal microscope (e.g. Yokogawa CSU-W1) with high definition EMCCD camera (e.g. Andor iXon Ultra 888)
3. Patch clamp amplifier (EPC-10 USB, HEKA) with a head stage attached to a micromanipulator
4. *Recording patch pipettes*: borosilicate glass capillaries with filament (1.5 mm OD/0.86 mm ID) pulled for a pipette resistance of 15-20 MΩ, tip heat-polished by a microforge, and coated with dental wax melted on a hotplate to minimize stray capacitance.

2.2. Culture media and solutions

1. Astrocyte-conditioned medium (ACM) (Wako)
2. *Plating culture medium (Medium 1)*: ACM supplemented with 2.5S nerve growth factor (100 ng/ml), brain-derived neurotrophic factor (25 ng/ml), fibroblast growth factor-2 (5 ng/ml), and KCl elevated to 25 mM.
3. *Culture medium (Medium 2)*: ACM supplemented with 2.5S nerve growth factor (100 ng/ml), NT-3 (100 ng/ml), brain-derived neurotrophic factor (25 ng/ml), fibroblast growth factor-2 (5 ng/ml), and KCl elevated to 25 mM.

4. *Standard artificial cerebrospinal fluid (aCSF)*: 125 mM NaCl, 2.5 mM KCl, 10 mM glucose, 1.25 mM NaH_2PO_4, 2 mM Na-pyruvate, 3 mM *myo*-inositol, 0.5 mM Na-ascorbate, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 1 mM $MgCl_2$. Stock solution (10x, excluding $CaCl_2$ and $MgCl_2$) can be stored at 4°C up to 2 weeks.
5. *Recording aCSF*: 10 mM tetraethylammonium chloride (TEA-Cl), 0.5 mM 4-aminopyridine, 1 µM tetrodotoxin, 10 µM bicuculline methiodide, 0.5 µM strychnine hydrochloride in aCSF. NaCl is replaced by TEA-Cl in order to adjust the osmolarity.
6. *Recording pipette solution*: 125 mM Cs-methanesulfonate, 20 mM CsCl, 10 mM HEPES, 0.5 mM EGTA, 12 mM Na_2-phosphocreatine, 3 mM MgATP, 1 mM $MgCl_2$, 0.3 Na_2GTP. Adjust pH to 7.3 with CsOH. Adjust osmolarity to 315-320 mOsm/kg with distilled H_2O or Cs-methanesulfonate. Na_2GTP should be added to the pipette solution on the day of use to avoid its breakdown. To label the patched presynaptic terminals (as in Fig. 7A), add 50 µM AlexaFluor-594 to the pipette solution.

3. Methods

3.1. Preparation of giant synapse culture

All experiments have been performed in accordance to the regulations of the Okinawa Institute of Science and Technology animal care and use committee (protocol #2015-128). Okinawa Institute of Science and Technology animal facilities and animal care and use program are accredited by AAALAC International (reference #1551).

1. In a tissue culture hood, add poly-D-lysine solution (MW > 300 kDa, 100 µg/ml, diluted in ultrapure H_2O) to completely cover the culture surface of dishes.
2. Leave the dishes for 1 hour in the tissue culture hood, and then wash 3 times with

ultrapure H_2O. After washing, allow dishes to dry for 1-3 hours (during the dissection procedure) with open lids in the tissue culture hood.

3. Prepare two 15 mL tubes with 2 mL Hank's Balanced Salt Solution (HBSS) and put on ice next to the dissecting stereomicroscope.
4. Collect a pup brain (P0-P1; C57BL/6 or ICR strains) and place it under a stereomicroscope on a HBSS-soaked filter paper circles (4-6 cm diameter) with ventral side facing up (Fig. 1A) (*see* **Note 1**).
5. Using forceps, grasp and remove the meninges from the brainstem region (Fig. 1B) making sure that all meninges are removed from the CN and the superior olivary complex regions.
6. Excise the CN region (Fig. 1C) by using one ophthalmic surgery blade to support the CN from under, and then the second blade to excise. Collect the pieces in a tube containing HBSS on ice.
7. Excise the MNTB (Fig. 1 C,D) region by making four ~1 mm incisions at the location of the MNTB region (blue outline in Fig. 1D) and scoop out a tissue block at 0.5~1 mm depth . Collect in a separate tube containing HBSS on ice.
8. After dissections, dissociate CN and MNTB tissues using a papain-based dissociation solution, or using a kit following the manufacturer's instructions (*see* **Note 2**). Process the two tubes containing the CN and MNTB pieces separately.
9. After dissociation, re-suspend each of the CN and MNTB cells in 1mL of Medium 1 in separate tubes and count the number using automatic cell counter (*see* **Note 3**).
10. Mix the two types of cell populations at 1:1 ratio, and plate a total density of 1.7-2.1 x 10^5 cells per 35 mm dish (0.75-0.8 mL medium volume per 35 mm Ibidi dish) in

Medium 1.

11. At culture day4 (DIV4) and every 4 days after that, exchange half of the medium (0.3-0.35 mL) with fresh Medium 2. During medium change at DIV8, supplement Medium 2 with cyctosine arabinoside-C (5 µM) to stop glial cell division.

3.2. Labeling and identification of giant synaptic terminals in culture

For identification of live calyceal terminals in the culture, we use 2 labeling methods ***(11)***, namely green fluorescent protein (GFP) over-expression in CN (presynaptic) neurons (Fig. 2) and FM dye labeling (Fig. 3). For most application, overexpression of cytosolic GFP is the best choice because it labels live terminals with minimal perturbation to synaptic function. Transfection of GFP by electroporation, and identification of giant synaptic terminals in culture are described below.

1. If CN cells are to be transfected, proceed with the following steps immediately after Point 9 in Section **3.1**.
2. In a sterile 15 mL tube, prepare the desired volume of Medium 1, depending on the number of dishes to be plated (0.75-0.8 mL medium volume per 35mm Ibidi dish).
3. Add the appropriate number of MNTB cells (~1 x 10^5 cells per dish) to the medium, and leave on ice.
4. In a sterile 1.5 mL tube, centrifuge (300 x g, 5 min) a portion of the CN cell suspension, and re-suspend cell pellet in electroporation buffer (*see* **Note 4**).
5. Adjust CN cell number with electroporation buffer to a cell count of 0.7-0.9 x 10^7 cells per mL.
6. Add GFP cDNA to cells at 0.8µg per 10 µL.
7. Mix well by tapping gently.

8. Leave the tube on ice for 10 minutes. In our hands, this step increases cell viability after electroporation.
9. Electroporate at 1350 V, 23 µs, 1 pulse (Neon electroporation system) using the 10 µL electroporation tip.
10. Add 10 µL of electroporated CN cells per each dish to the 15 mL tube containing Medium 1 and MNTB cells.
11. Mix the electroporated CN cells with the MNTB cells by pipetting gently and plate on culture dishes.
12. Culture for 15-21 days as described in Section **3.1** (*see* **Note 5**). Change medium every 4 days as described in Section **3.1**, Point 11.
13. For identifying giant terminals in culture, we use a Apochromat 40x, 1.2NA water immersion objective and epifluorescence microscopy. GFP-expressing calyceal giant terminals can be identified by two main visual cues - first, as ring-like structures surrounding a non-fluorescent cell (Fig. 2A and B), and second, finger-like structures (covered with swellings like beads on a string) extending form the ring upward and covering the entire non-fluorescent cell (Fig. 2B**) (** *see* **Note 6**).

3.3. Labeling and live imaging of synaptic vesicle dynamics

In this protocol, synaptic vescicles from cultured giant terminals are labeled with rabbit polyclonal antibodies directed against the intravesicular (lumenal) domain of synaptotagmin-2 (Syt2) tagged with either quantum dots Q655, or with the pH-sensitive fluorophore CypHer5E (C5E) as reported in Guillaud et al. ***(17)***.

1. In a total volume of 10 µL, incubate 1 µg of Syt2 antibody with 2.5 µg of secondary goat anti-rabbit F(ab)'$_2$ antibody (conjugated with quantum dots Q655 or with CypHer5E) for 30 to 45 minutes at room temperature (*see* **Note 7)**.
2. Add 90 µL of fresh culture medium and directly apply to primary cultures between DIV18 and DIV21, and incubate at 37° C and 5% CO_2 for 1-16 hours (*see* **Note 8**).
3. Place culture dish on the stage of confocal laser scanning microscope equipped with a temperature-controlled Plan-Apochromat 63x, 1.45 NA oil immersion lens.
4. Before imaging, replace culture medium with Tyrode's salt solution (pH = 7.4) by continuous perfusion through a peristaltic pump connected to a temperature controller, set to maintain a constant physiological temperature of 36.5° C throughout the imaging session.
5. Localize giant presynaptic terminals over-expressing cytosolic GFP (as described in Section **3.2**) and loaded with Q655-Syt2 or C5E-Syt2, and define a region of interest preferentially located in the upper region of the giant synaptic terminal and containing several interconnected presynaptic swellings (Fig. 4). Adjust image pixel resolution and scanning speed to achieve an image acquisition rate of ~1 Hz, and acquire time series for 60-120 seconds. For single 2D optical section, use initial image resolution 512 x 512 pixels, pixel dwell time 3.15 µs. For 3D tracking, acquire 4 optical sections (~300 nm) covering the height of presynaptic swellings (~1.5 µm) with a pixel dwell time of 1.27 µs.
6. After acquisition, apply a median filter algorithm to raw confocal data.
7. Perform fluorescence spot detection and tracking on 30-second image sequences using the autoregressive motion algorithm with an initial spot size of 150 nm

(Gaussian fitted) and a maximum distance between spots on 2 consecutive frames of 0.8 μm, without frame gap ***(17)***.

8. Synaptic vesicle movements and trajectories (speed, trajectory length, mean square displacement curves, diffusion coefficient, etc.) can be analyzed with the IMARIS software package (Fig. 5**) (***see* **Note 9**).

3.2 Visualization and quantification of vesicle exocytosis

1. Apply C5E-Syt2 solution to primary cultures and load synaptic vesicles as described in Section **3.3**.
2. Perform chemical stimulation by perfusing Tyrode's salt solution containing 65 mM KCl and acquire time series as in Section **3.3**.
3. Measure and analyze the fluorescence intensity signal from C5E-Syt2 over time during KCl stimulation. Upon stimulation, exocytosis of synaptic vesicles induces an 80% decrease in C5E signal, indicative of massive exocytosis (**Fig. 6) (***see* **Note 10**).

3.3 Membrane capacitance measurement

1. Transfer a culture dish with GFP-labeled cultures (as described in Section **3.2**) to the recording chamber under the objective, and perfuse standard aCSF to the dish (~2 mL/min) warmed to 37°C.
2. Place reference electrode into the dish.
3. Using epifluorescence, identify a GFP-expressing giant calyceal terminal (as described in Section **3.2**) with presynaptic swellings (1-3 μm diameter; *see* **Note 11**).
4. Upon identification of a suitable giant synaptic terminal, change perfusion solution to recording aCSF.

5. Load pipette solution into a recording patch pipette, and gently tap the pipette to remove air bubbles from the tip.
6. Set the pipette into the headstage of patch clamp amplifier. Apply positive pressure (>2 psi) to the pipette, and dip it into the recording aCSF in the dish. Find the tip of the pipette under transmitted light (*see* **Note 12)**.
7. Position the recording pipette above the calyceal terminal. Release pressure from the pipette once, and then apply weak pressure (~0.5 psi) again.
8. Observe the presynaptic swelling by GFP fluorescence, and approach the pipette to the swelling of cultured calyces (*see* **Note 13).**
9. Form a Giga-ohm seal by applying gentle negative pressure. Adjust electrode capacitance, and then achieve a whole-cell configuration. If Alexa dye is added to the recording pipette solution, it will diffuse into calyceal terminals within 5-10 minutes **(Fig. 7A) (***see* **Note 14).**
10. Set whole-cell parameters (cell capacitance and series resistance) and compensate series resistance up to 70% for a final value of 15 MΩ. The cell capacitance of cultured calyceal terminals is 3-5 pF.
11. Set holding potential of -80 mV.
12. Apply a sinusoidal voltage command (peak-to-peak voltage of 60 mV at 1 kHz) and record current. The recorded current is then converted to membrane capacitance (C_m), membrane conductance (G_m) and series conductance (G_s) by the Lock-In Amplifier software. C_m jump caused by vesicle exocytosis can be induced by either a single or a train of depolarizing pulse(s) (Fig. 7). To avoid contamination of G_m-dependent

capacitance artifacts, C_m changes within 450 ms of stimulation should be excluded from analysis (***19***).

4. Notes

1. Perform the whole procedure (removing brain, and dissection regions immediately) for each brain and keep dissected regions on ice in HBSS. Alternatively, remove all brains first and keep in HBSS on ice, then proceed with dissecting CN and MNTB regions. Both give similar results, but performing whole procedure for each brain is faster overall.
2. Commercially available neuronal cell dissociation kits work best in our experience, but separate reagents can also be used. If separate reagents are used, standard protocols for dissociation and preparation of hippocampal neuronal cell culture can be followed.
3. Usually, the number of dissociated MNTB cells is ~32,000 cells per pup, and for dissociated CN cells ~160,000 cells per pup. If cell numbers differ by more than 2-fold (especially for the MNTB region), then the dissection procedure was not optimal. If much higher cell numbers (3-10 x) are obtained, then the dissected area was too big and other cells from the superior olivary complex were collected as well, or vice versa. In such case, adjust the dissection area accordingly.
4. From two mouse litters, usually taking 0.5 ml of CN cells for centrifugation and re-suspending in 0.1 mL of electroporation buffer will result in approx. 0.9 x 10^7 cells per mL. Use that as a starting point and then adjust with electroporation buffer. If fewer dishes are to be plated adjust accordingly.

5. For live imaging and patch clamp experiments, DIV 18-20 cultures are best in our experience.
6. We usually look for the giant terminals using epifluorescence and first search for the ring structure surrounding a healthy non-fluorescent cell body. Once we find the ring-like structure, we focus on top of the non-fluorescent cell body to look for the finger-like structures.
7. Alternatively, it is possible to directly label Syt2 primary antibody using SiteClick™ Qdot® 655 Antibody Labeling Kit (Thermo Fisher) or use primary antibody against Syt2 pre-conjugated with C5E (Synaptic System). If Syt2 pre-labeled antibodies are to be used, omit step 1.
8. Depending on the incubation period, the total number of loaded vesicles can be adjusted from sparse labeling (1 hour) to dense labeling (overnight).
9. Other publically and freely available software could be used for tracking and analysis, such as Icy bio-imaging software (http://icy.bioimageanalysis.org/).
10. Visualization and quantification of endocytosis can also be achieved by loading Syt2-C5E into vesicles and applying electrical stimulation at 1 Hz (80-100 mV), which induces a significant increase in the number of loaded vesicles as well as significant increase in their fluorescence intensity due to the acidification of the vesicles after their retrieval from plasma membrane.
11. To enlarge the images of the patch region, a high-resolution digital camera (e.g. Axiocam 505 mono, Zeiss) and/or an intermediate magnification changer is required.

12. Coating the tip of pipettes with Alexa 488-conjugated bovine serum albumin may be helpful for visualizing the pipette tip while approaching the presynaptic swellings ***(20)***.
13. When the pipette is close enough to the swelling, slight deformations of the swelling surface can be observed caused by the stream of recording solution flowing out of the patch pipette (because of the positive pressure applied to the pipette).
14. Too much negative pressure might cause the swelling to be sucked into the pipette. If it is difficult to rupture the patch membrane by pressure, rupture it by zapping.

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Figure Legends

Figure 1. Dissection locations for preparation of giant synapse culture. (A) P1 mouse brain placed on a HBSS-soaked filter paper with ventral side facing up. **(B)** Same brain as in *A*, with meninges removed from the brainstem region (arrow). **(C)** Magnification of the brainstem region with the cochlear nuclei (CN) and medial nucleus of the trapezoid body labeled (MNTB). **(D)** Magnification of the brainstem region with labeled pontine nuclei region, midline (dotted line) and the MNTB excision area (blue lines).

Figure 2. Imaging and identification of live giant synaptic terminals by GFP over-expression. (A) Left, schematic depicting a GFP-expressing neuron, contacting and forming a giant terminal onto a non-fluorescent cell body. Right, confocal images of three giant presynaptic terminals expressing cytosolic GFP (green). In these examples, the terminals are visible without the GFP labeling (arrowheads). **(B)** Confocal images (GFP fluorescence - top; GFP and DIC - bottom) of two terminals visible as ring structures at lower confocal plane (left; z=3 μm) and extending finger-like processes upward (middle, z=6 μm), and covering the whole non-fluorescent cell body (right, z=9 μm). Scale bar is 10 μm.

Figure 3. Imaging and identification of live giant synaptic terminals by FM dye loading. (A) Confocal image of live giant terminals (arrows) loaded with FM 4-64 dye (red) and DIC image (right). **(B)** Higher magnification confocal image of live giant terminals loaded with FM 4-64 dye (red) and merged DIC imaged (bottom). Scale bar is 10 μm.

Figure 4. Imaging and detection of labeled synaptic vesicles. Confocal z-stack imaging of a giant presynaptic terminal expressing cytosolic GFP- (green) and Q655-Syt2 (red)-labeled vesicles, and corresponding volume-rendering of the GFP fluorescence with synaptic vesicle spot detection (reproduced from Guillaud, L., Dimitrov, D., Takahashi T. (2017) Presynaptic morphology and vesicular composition determine vesicle dynamics in mouse central synapses. eLife 2017;10.7554/eLife.24845. © Guillaud et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License).

Figure 5. Trajectory analyses of labeled synaptic vesicles. Live confocal imaging of a giant calyceal terminal expressing cytosolic GFP- and Q655-Syt2-labeled vesicles tracking sorted according to their trajectory lengths (blue < 2 μm, green 2-4 μm and red > 4 μm). Scatter plot of vesicle trajectory lengths and maximum speeds superimposed with individual trajectory traces color-coded (reproduced from Guillaud, L., Dimitrov, D., Takahashi T. (2017) Presynaptic morphology and vesicular composition determine vesicle dynamics in mouse central synapses. eLife 2017;10.7554/eLife.24845. © Guillaud et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License).

Figure 6. Visualization and quantification of C5E-Sty2-labeled vesicle exocytosis induced by bath application of 65 mM KCl. Upper left panel: confocal image before KCl application, lower left panel: confocal image after KCl application. Right panel: Measurement of C5E fluorescence intensity from region of interest (white box on left panels) of 5 different terminals. Black trace: average of 5 traces (color coded), Red trace: Boltzman fitting (reproduced from Guillaud, L., Dimitrov, D., Takahashi T. (2017)

Presynaptic morphology and vesicular composition determine vesicle dynamics in mouse central synapses. eLife 2017;10.7554/eLife.24845. © Guillaud et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License).

Figure 7. Whole-cell patch clamp recording from cultured calyceal terminals. (**A**) Left, DIC image of patch clamp recording from a cultured calyceal terminal with recording pipette. Middle, GFP epifluorescence image of a cultured calyceal terminal. Right, epifluorescence image of Alexa-fluor 594 injected into a calyceal terminal via patch pipette. Scale bar is 10 µm. Inset shows a zoom of a patched swelling of the cultured terminal (scale bar is 2 µm). (**B**) Membrane capacitance changes of cultured calyceal terminals. C_m jump was induced by a single 20-ms depolarizing pulse (from -80 to +10 mV, n = 4, left) or a train of 20-ms pulse at 20 Hz (20 times, n = 5, right). Error bars indicates S.E.M. (**C**) Normalized traces of membrane capacitance measurements (black trace) combined with simultaneous imaging of pHluorin signals (red trace).