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TITLE:

Presynapse Formation Assay Using Presynapse Organizer Beads and "Neuron Ball" Culture

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SUMMARY:

Presynapse formation is a dynamic process including accumulation of synaptic proteins in proper order. In this method, presynapse formation is triggered by beads conjugated with a presynapse organizer protein on axonal sheets of "neuron ball" culture, so that accumulation of synaptic proteins is easy to be analyzed during presynapse formation.

ABSTRACT:

During neuronal development, synapse formation is an important step to establish neural circuits. To form synapses, synaptic proteins must be supplied in appropriate order by transport from cell bodies and/or local translation in immature synapses. However, it is not fully understood how synaptic proteins accumulate in synapses in proper order. Here, we present a novel method to analyze presynaptic formation by using the combination of neuron ball culture with beads to induce presynapse formation. Neuron balls that is neuronal cell aggregates provide axonal sheets far from cell bodies and dendrites, so that weak fluorescent signals of presynapses can be detected by avoiding overwhelming signals of cell bodies. As beads to trigger presynapse formation, we use beads conjugated with leucine-rich repeat transmembrane neuronal 2 (LRRTM2), an excitatory presynaptic organizer. Using this method, we demonstrated that vesicular glutamate transporter 1 (vGlut1), a synaptic vesicle protein, accumulated in presynapses faster than Munc18-1, an active zone protein. Munc18-1 accumulated translation-dependently in presynapse even after removing cell bodies. This finding indicates the Munc18-1 accumulation by local translation in axons, not transport from cell bodies. In conclusion, this method is suitable to analyze accumulation of synaptic proteins in presynapses and source of synaptic proteins. As neuron ball culture is simple and it is not necessary to use special apparatus, this method could be applicable to other experimental platforms.

INTRODUCTION:

Synapse formation is one of critical steps during development of neural circuits[1–3]. Formation of synapses that are specialized compartments composed of pre- and post-synapses is a complex

and multistep process involving appropriate recognition of axons and dendrites, formation of active zone and postsynaptic density, and proper alignment of ion channels and neurotransmitter receptors[1,2]. In each process, many kinds of synaptic proteins accumulate to these specialized compartments in proper timing by transporting synaptic proteins from cell bodies and/or by local translation in the compartments. These synaptic proteins are considered to be arranged in organized manner to form functional synapses. Dysfunction of some synaptic proteins involving synapse formation results in neurological diseases[4,5]. However, it remains unclear how synaptic proteins accumulate in proper timing.

To investigate how synaptic proteins accumulate in organized manner, it is necessary to examine accumulation of synaptic proteins in chronological order. Some reports demonstrated live imaging to observe synapse formation in dissociated culture of neurons[6,7]. However, it is time-consuming to find neurons which just start synapse formation under microscopy. To observe accumulation of synaptic proteins efficiently, synapse formation must start at the time when researchers want to induce the formation. The second challenge is to distinguish accumulation of synaptic proteins due to transport from cell bodies or local translation in synapses. For that purpose, translation level is necessary to be measured under the condition that does not allow transport of synaptic proteins from cell bodies.

We developed novel presynapse formation assay using combination of neuron ball culture with beads to induce presynapse formation[8]. Neuron ball culture is developed to examine axonal phenotype, due to the formation of axonal sheets surrounding cell bodies[9,10]. We used magnetic beads conjugated with leucine-rich repeat transmembrane neuronal 2 (LRRTM2) that is a presynaptic organizer to induce excitatory presynapses (**Figure 1A**)[11–13]. By using the LRRTM2 beads, presynapse formation start at the time when the beads are applied. This means that presynapse formation starts in thousands of axons of a neuron ball at same times, thus it allows to examine precise time course of accumulation of synaptic proteins efficiently. In addition, neuron ball culture is easy to block transport synaptic proteins from soma by removing cell bodies (**Figure 1B**)[8]. We have already confirmed that axons without cell bodies can survive and are healthy at least 4 h after removal of cell bodies. Thus, this protocol is suitable to investigate from where synaptic proteins are derived (cell body or axon), and how synaptic proteins accumulate in organized manner.

PROTOCOL:

The experiments described in this manuscript were performed according to the guidelines outlined in the Institutional Animal Care and Use Committee of the Yokohama City University.

1. Preparation of neuron balls as hanging drop culture (Days *in vitro* (DIV) 0-3)

NOTE: The procedures described here for the preparation of neuron ball culture are based on the method previously reported by the Sasaki group with some modifications[9,10]. We also adopted several procedures from the Banker method for dissociated culture[14].

1.1. Confirming the followings before starting the dissection

1.1.1. Prepare all the required solutions and sterilize them by autoclave/filtration in advance.

1.1.2. Keep ready all the instruments and materials to be used in each steps of this cortical neuron culture.

1.1.3. Spray and wipe the laminar air flow cabinet, dissection table, stage plate of stereomicroscope, scissors, and forceps with 70% ethanol.

1.2. Euthanize the mouse upon application of CO_2 and dissect the abdomen to obtain E16 embryos.

1.3. Remove the brains from embryos carefully with the help of fine tips of forceps and transfer them into 60 mm cell culture dishes containing 4 mL of HEPES Buffered Salt Solution (HBSS).

NOTE: The dissection medium HBSS contains 10 mM HEPES (pH 7.4), 140 mM NaCl, 5.4 mM KCl, 1.09 mM Na_2HPO_4, 1.1 mM KH_2PO_4, 5.6 mM D-glucose, and 5.64 µM phenol red.

1.4. Remove the scalp, cut the olfactory bulb, separate cortices from each cerebral hemisphere using the fine tips of forceps under stereomicroscope, and transfer to another 60 mm dishes containing fresh HBSS. Use at least 3-5 embryos for each separate neuron ball culture.

1.5. Cut the cortices into small pieces with microdissecting spring scissors in a laminar flow cell culture hood.

1.6. Transfer minced cortices to a 15 mL tube and trypsinize the minced cortices in 4 mL of 0.125% trypsin in HBSS for 4.5 min in a water bath at 37 °C.

NOTE: This trypsinization time is critical for efficient neuron culture as the increasing time (> 4.5 min) leads to much more dead neurons.

1.7. Transfer the cell aggregates to a new 15 mL tube containing 10 mL of HBSS by a sterile transfer pipette, and incubate at 37 °C for 5 min. Repeat this step one more time.

1.8. Transfer the cell aggregates to a new 15 mL tube containing 2 mL of Neurobasal media containing GlutaMax, B27 supplement (NGB medium), 0.01% DNase I and 10% horse serum.

1.9. Triturate the trypsinized cortices by repeatedly pipetting them up and down (3-5 times) using fire-polished fine glass Pasteur pipette.

NOTE: Diameter of fire-polished fine glass Pasteur pipette is very important as described in the Banker method paper[14]. If the pipette is too narrow to pass cortices, prepare pipettes possessing 2-3 different sizes, and try from larger pipette.

1.10. For preparing neuron balls, take the above cell suspension and adjust the cell density to 1 x 10^6 cells/mL using NGB medium.

1.11. Culture the cortical neurons as hanging drops containing 10,000 cells/drop (1 drop is 10 µL) inside the upper lids of 10 cm culture dishes.

1.12. Add 7 mL of phosphate buffered saline (PBS) to the bottom part of culture dishes, then keep in an incubator for 3 days at 37 °C with 5% CO_2 under humidified condition to allow for neuron ball formation.

2. Placing neuron balls on PLL-coated glass coverslips and culture maintenance (DIV 3-11)

NOTE: Before coating of glass coverslips with poly-L-lysine (PLL), washing the coverslips using detergent is important. Glass coverslips are sometimes not so clean for neuronal culture and uniform coating with PLL. Non-uniform PLL coating may result in uneven axonal extension of neuron balls.

2.1. Soak the coverslips in 1/20 diluted neutral non-phosphorous detergent in ceramic racks for 1-3 overnights.

2.2. Wash the coverslips 8 times in ultrapure water, and then sterilize in an oven at 200 °C for 12 h.

NOTE: All the steps from here must be done in a laminar air flow cell culture hood.

2.3. Transfer baked coverslips to 100-mm dishes. After sealing the dish by parafilm between a lid and a bottom dish, baked coverslips can be kept for long-term storage.

2.4. (Optional) Apply paraffin dots to the coverslips. The dots make space to prevent neuron balls detaching from the coverslips during immunostaining by direct contact of neuron balls to plastic dishes. Melt paraffin in a suitable bottle in a hot water bath at about 90 °C. Dip a Pasteur pipette into the paraffin, then rapidly touch it to make three spots near the edge of a coverslip.

2.5. Coat PLL (MW > 300,000) onto the paraffin-beaded glass coverslips in 60-mm dishes using PLL solution (15 µg/mL in borate buffer), and keep them for at least 1 h in a CO_2 incubator.

2.6. After washing 4 times with PBS, transfer the PLL-coated coverslips to a 4-well plate containing 350 µL of NGB medium in each well and cytosine β-D-arabinofuranoside hydrochloride (AraC, 3 µM) to the media to kill dividing cells.

2.7. Keep this 4-well plate containing the PLL-coated coverslips in the CO_2 incubator at least for 20 min to ensure the temperature of the medium reach 37 °C before transferring neuron balls.

2.8. At DIV 3 when “neuron balls” are formed very well, transfer them onto PLL-coated coverslips inside the 4-well plate (5 neuron balls/well) kept in the CO_2 incubator.

2.9. After 48 h, replace the neuron ball culture medium with fresh AraC-free NGB medium. Use a hot plate in a laminar air flow cell culture hood whose temperature is kept ready at 37 °C immediately before this procedure.

NOTE: It is necessary to perform the medium changing as rapidly as possible on a hot plate to reduce the time that the cultures are at the outside of the CO_2 incubator.

2.10. Keep this neuron ball culture in the CO_2 incubator for up to DIV 11.

NOTE: At DIV 11-12, neuron balls extending neurites up to 1-2 mm are used for experiments.

3. Applying LRRTM2 beads on neuron ball culture with or without cell bodies (DIV 11-12)

NOTE: Before application of LRRTM2 beads on neuron ball culture, it is recommended to remove cell bodies. Therefore, prepare LRRTM2 beads at first, then remove the cell bodies and later apply LRRTM2 beads to culture as early as possible. Biotinylated LRRTM2 is provided by the Nogi’s group (Yokohama City University) as conditioned medium. They use an expression vector including biotin acceptor sequence and biotin ligase from *E. coli* (BirA)[15,16] to attach biotin to LRRTM2, and the expression vector is transfected to Expi293F cells included in the Expi293 Expression System. The vector information is available in **Supplementary Figure 1**. Biotinylated LRRTM2-conjugated streptavidin beads reduced background of immunostaining greatly compared to LRRTM2-Fc –conjugated Protein A beads that are used for prototype LRRTM2 system[8].

3.1. Preparation of biotinylated LRRTM2 beads

3.1.1. To remove excess biotin from conditioned medium of Expi293F cells expressing biotinylated LRRTM2, apply 1.7 mL of the conditioned medium mixed with 0.8 mL of PBS (total 2.5 mL) to PD-10 gel filtration column. PD-10 column is pre-equilibrated with 25 mL of ultrapure water and 25 mL of PBS.

3.1.2. Elute with 3.5 mL of PBS and collect the flow-through as a LRRTM2 stock.

NOTE: This LRRTM2 stock can be dispensed to aliquots and stored at -80 °C for long-term. Expression levels of biotinylated LRRTM2 sometimes vary from lot to lot. Thus, proper volume of LRRTM2 stock to conjugate to the beads should be determined to form presynapses enough on neuron balls, when new lot of LRRTM2 stock is used at first time.

3.1.3. Take 20 µL from the suspension of streptavidin-coated magnetic particles (diameter: 4-5 µm) to a microcentrifuge tube. Immobilize the beads to a handmade apparatus attached with

neodymium permanent magnets and wash three times with 100 µL of PBS-MCBC in 1.5 mL microcentrifuge tubes.

NOTE: PBS-MCBC contains PBS including 5 mM $MgCl_2$, 3 mM $CaCl_2$, 0.1% BSA, and 0.1% Complete EDTA-free.

3.1.4. After removing completely PBS-MCBC from the beads, add predetermined volume of LRRTM2 stock (usually 500-1,000 µL, see Note 3.1.2) to the washed beads. Incubate the mixture using rotator at 4 °C for 1-2 h.

3.1.5. Wash the beads twice with 100 µL of PBS-MCBC, and later with 100 µL of NGB medium.

3.1.6. Resuspend the LRRTM2 beads in 50 µL of NGB medium for application to the neuron ball culture.

3.1.7. Use same procedures for preparing the control beads (negative control) in another microcentrifuge tube except adding biotinylated-LRRTM2 proteins.

3.2. Removing cell bodies from neuron balls at DIV 11-12 and applying LRRTM2 beads

3.2.1. Label the wells of a 4-well plate as “Cell body (+)” and “Cell body (-)”.

3.2.2. Cut the ending of a yellow tip at 45° angle with a razor blade previously sprayed with 70% ethanol under stereomicroscope (**Figure 1B**).

3.2.3. Put the yellow tip end on the cell body area of a neuron ball and remove the cell bodies by suction (**Figure 1B**).

3.2.4. To identify each specified condition of the experiment, label the wells again as “LRRTM2 beads” and “Control beads”.

3.2.5. Apply the LRRTM2 and control beads on neuron ball culture, and submerge to the bottom of the plates for 1 min using ferrite magnets to start presynapse formation. This procedure ensures to touchdown all beads at the same time. Especially, it would be effective for short time incubation (e.g., 30 min and 1 h) with LRRTM2 beads synchronously.

3.2.6. To perform time-course experiments, label each separate well as 0 min, 30 min, 1 h, 2 h, 4 h and 18 h and apply LRRTM2 beads at the indicated time intervals.

3.2.7. After the addition of LRRTM2 beads, incubate the neuron ball culture with the beads for specified time (0 min to 18 h) to form presynapses.

4. Immunostaining and microscopy

NOTE: Fix the cells for 4 h via incubation with LRRTM2 beads in the experimental conditions with and without cell bodies, as the axons tend gradually to die in absence of cell bodies after 4 h. In case of time course with LRRTM2 beads, fix the cells at the indicated specified time.

4.1. Fixing and staining neurons in neuron ball culture after presynapse formation with LRRTM2 beads

4.1.1. Remove NGB medium and fix the neuron balls with 4% PFA in PBS (250 µL/well) for 20 min at room temperature, and then wash with 500 µL of PBS 4-times each 5 min.

4.1.2. Wash the fixed cells with TBS (Tris-buffered saline: 50 mM Tris-HCl (pH 7.3) and 150 mM NaCl) for more than 5 min.

4.1.3. Permeabilize the cells/axons of neuron ball culture with TBS containing 0.3% Triton X-100 for 5 min.

4.1.4. Keep the cells 1 h for blocking with blocking buffer (0.1% Triton X-100 and 5% NGS (normal goat serum) in TBS).

4.1.5. Incubate the cells with primary antibodies; anti-rabbit-vGlut1 (vesicular glutamate transporter 1 (1:4000)) and anti-mouse-Munc18-1 (1:300) diluted with antibody diluent for overnight at 4 °C.

4.1.6. Wash the coverslips 4 times with immunofluorescence (IF) buffer (0.1% Triton X-100 and 2% BSA in TBS) and incubate for 30 min with fluorophore (Alexa dye)-conjugated 2nd antibodies; anti-mouse-IgG-Alexa 555 (1:500), anti-rabbit-IgG-Alexa 488 (1:1000).

4.1.7. Stain the nuclei of the cell bodies of neurons for 5 min with 4′,6-diamidino-2-phenylindole (DAPI, 1 µg/mL) in PBS.

4.1.8. Wash the coverslips three times with PBS and then mount on glass slides using mounting media containing 167 mg/mL poly (vinyl) alcohol and 6 mg/mL N-propyl gallate.

4.1.9. Store the glass slides in a refrigerator at -20 °C until microscopy. Fluorescent signals of the glass slides are detectable for at least 1 year when the slides are stored at -20 °C.

4.2. Taking images under fluorescence microscope

4.2.1. Capture phase and IF images under an inverted fluorescent microscope with a cooled CCD camera using 60X oil immersion lens. For image acquisition software, use a software installed microscope system. Use Image J as image analysis software.

4.2.2. Measure the IF intensity in presynapses in axon using following formula; IF intensities of Region of Interest on beads (ROI) – Off beads region intensity/Axonal intensity along 20 µm from

beads – background intensity. This ratio intensity provides protein accumulation index (**Figure 4A**). Accomplish the measurements on original 16-bit images using Image J software.

4.2.3. To quantify the accumulation level of particular protein in presynapse induced with LRRTM2 beads, always select the area away from 2-field of view or more apart from the cell body with microscope (60X).

NOTE: The selection of area in neuron ball for imaging is crucial as dense axons are near the cell body and periphery of neuron ball can provide single axon.

4.2.4. For accurate measurement, choose 5-different axonal field (similar distance from cell bodies)/coverslip.

4.2.5. Keep identical imaging conditions at different day and in between experiments

REPRESENTATIVE RESULTS:

Here, we show representative results of accumulation of presynaptic proteins in LRRTM2-induced presynapses of axonal sheets of neuron ball culture. As presynaptic proteins, we analyzed the excitatory synaptic vesicle protein vGlut1 and the active zone protein Munc18-1. We also examined time course of accumulation of vGlut1 and Munc18-1 in presynapses, and obtained results indicating source of Munc18-1 in presynapses using axons removing cell bodies and a protein synthesis inhibitor. Recently, we have investigated a role of Fragile X mental retardation protein (FMRP) on accumulation of Munc18-1 in presynapses[8]. FMRP, which is a causative gene product of Fragile X syndrome (FXS), is a mRNA binding protein to suppress translation[17–19]. We also examined the involvement of FMRP in the Munc18-1 accumulation using FXS model mice which is deficient in *Fmr1* gene encoding FMRP.

Application of LRRTM2-beads into neuron ball culture at DIV11 induced accumulation of Munc18-1 in presynapses of axons of neuron balls (**Figure 2A**). Even in axons which are removed cell bodies, accumulation of Munc18-1 was observed under the beads similar as axons of neuron balls with cell bodies (**Figure 2B**). In typical case, over 80% of beads after 4 h-incubation with neuron balls can induce accumulation of synaptic proteins in presynapses, judged by staining Munc18-1 and vGlut-1. Because axons are so crowded and overlapped near cell bodies (**Figure 2Aa, Ba**), more peripheral region of axonal sheets were measured where axons are not so overlapped (**Figure 2Ab, Bb**, e.g., peripheral area away from 2-field of view or more apart from the cell body with microscope (60X)). When peripheral region of axonal sheet was analyzed by high-magnification objective lens (60X), vGlut1 and Munc18-1 accumulated clearly in presynapses of axons under the beads (**Figure 3**). Sometimes, fluorescent signals of synaptic vesicular proteins like vGlut1 are hard to be detected in axonal region outside the beads, because these synaptic vesicular proteins accumulate so much under the beads. In the case of Munc18-1, fluorescent signals can be detected weakly in axonal region outside the beads.

To quantify accumulation level of synaptic proteins in presynapses induced by LRRTM2-beads, fluorescent intensities of axons under the beads and outside the beads were measured, and then

calculated as “Protein accumulation index” (**Figure 4A**, and described in protocol section in detail). Time course experiments demonstrated that accumulation of vGlut1 in presynapses increased significantly at 30 min (**Figure 4B**). On the other hand, Munc18-1 accumulation started to increase significantly at 2 h, and reach a plateau at 4 h (**Figure 4C**). These data indicate that the synaptic vesicle protein vGlut1 accumulates in presynapses earlier than the active zone protein Munc18-1. The Munc18-1 accumulation in presynapses of *Fmr1*-KO neurons increased 1.5 times more than those in wild type (WT) (**Figure 4C**), indicating involvement of FMRP in Munc18-1 accumulation. Next, to distinguish the Munc18-1 accumulation due to transport from cell bodies or local translation in axons, an effect of the protein synthesis inhibitor anisomycin were examined on the accumulation in the presence or absence of cell bodies (**Figure 4D**). Anisomycin suppressed the Munc18-1 accumulation significantly in axons (**Figure 4D**), indicating that the accumulation is protein synthesis-dependent. The accumulation in presynapses of axons without cell bodies was not significantly different to that with cell bodies (**Figure 4D**). These results suggest that accumulation of Munc18-1 in presynapses are derived mostly from axons, but not transport of Munc18-1 from cell bodies. If accumulation of synaptic proteins is suppressed in presynapses of axons by removing cell bodies, it is considered that this decrease is due to transport from cell bodies. Actually, when we examined the accumulation of total newly synthesized proteins metabolically labeled by fluorescent dye, removing cell bodies reduced significantly the accumulation of total newly synthesized proteins, compared to presynapses of axons with cell bodies[8]. Although the Munc18-1 accumulation in *Fmr1*-KO increased more compared to WT, anisomycin suppressed the accumulation in similar level to WT and removing cell bodies had no effect on the accumulation (**Figure 4D**). These results suggest that FMRP is involved in local translation of Munc18-1 in axons.

Representative results presented here demonstrate that this method is suitable to investigate how synaptic proteins accumulate in organized manner by time course experiments, and to examine source of synaptic proteins (transport from cell bodies or local translation in axons) by removing cell bodies.

FIGURE AND TABLE LEGENDS:

Figure 1. Scheme of presynapse formation and removal of cell bodies from neuron ball. (A) Presynapse formation assay using biotinylated LRRTM2 conjugated streptavidin beads to induce the presynapses in axons of neuron ball culture prepared from E16 cortices. LRRTM2, a postsynaptic protein, binds neurexin (NRXN) and act as a presynapse organizer. Streptavidin beads conjugated to biotinylated LRRTM2 extracellular regions (LRRTM2 beads) were applied at DIV11-12 to neuron ball culture to induce presynapses. This figure has been modified from previous publication[8]. **(B)** The yellow tip end was cut and placed on the cell body area of neuron ball culture at 45° angle. The cell bodies were removed by suction.

Figure 2. Munc18-1 accumulation in presynapses in presence and absence of cell bodies of neuron ball. (A) After 4 h incubation with LRRTM2 beads, the active zone protein Munc18-1 accumulated at the induced presynaptic sites in axons (Upper panel; experimental scheme, middle; phase image, lower; IF images of Munc18-1 accumulation). Images were captured as low magnification images using 10X lens and intermediate magnification (1.5X) to see the whole

picture composed of a neuron ball, axons, and beads. Dashed squares indicate the area of neuron ball for accurate imaging position of beads. Scale bar, 20 µm (left; original image), 10 µm (right; enlarged image). **(B)** Munc18-1 accumulated very well even in the absence of cell bodies at the induced presynaptic sites in axons. This result indicates that axons can survive and form presynapses even after removing cell bodies for at least 4 h.

Figure 3. Accumulation of vGlut1 and Munc18-1 in presynapses of axons with and without cell bodies. The excitatory presynaptic marker vGlut1 (green) and Munc18-1 (Red) accumulated in presynapses 4 h after addition of LRRTM2 beads. (Upper panel; with cell bodies, lower; without cell bodies). Images were captured using 60X oil immersion lens for high magnification to measure fluorescent intensity. Dashed circle outlined the position of beads. Munc18-1 accumulated almost similar extent in presence and absence of cell bodies in presynapses but vGlut1 accumulation is reduced without cell bodies[8]. Scale bar, 5 µm.

Figure 4. Procedure of IF intensity measurement and impact of cell bodies removal and protein synthesis inhibitor on Munc18-1 accumulation in neuron balls. (A) Diagram showed the quantification method of IF intensity at an induced-presynaptic site in axon of neuron ball culture. Scale bar, 5 µm. The details are described in protocol section. **(B)** Time course of vGlut1 accumulation in presynapses induced with LRRTM2 beads. Data shown are mean ± SEM for n = 20. Two-way ANOVA with Tukey's multiple comparison test. $^{**}p < 0.01$. **(C)** Time course of Munc18-1 accumulation in WT and *Fmr1*-KO presynapses under LRRTM2 beads. Data shown are mean ± SEM for n = 20, Two-way ANOVA with Tukey's multiple comparison test. n.s., not significant; $^{**}p < 0.01$, significantly different between WT and KO. **(D)** The bar graph showed Munc18-1 accumulation level in presynapses of WT and *Fmr1*-KO neuron balls in the presence or absence of 25 µM anisomycin (Aniso) with (CB+) or without (CB-) cell bodies. Data shown are mean ± SEM for n = 20. Two-way ANOVA with Tukey's multiple comparison test. $^{**}p < 0.01$, n.s., not significant. # indicated $p < 0.01$, significantly different with and without anisomycin. These figures have been modified from previous publication[8].

Supplementary Figure 1. Bicystronic expression vector for LRRTM2-ECR (Extracellular Region) and BirA (biotin ligase from *E. coli*). Between LRRTM2-ECR and BirA coding sequences, there is an Internal Ribosomal Entry Site (IRES) sequence that allows to co-express both proteins from single mRNA. hEF1-HTLV promoter drives expresson the bicstronic mRNA, and both proteins are secreted by signal peptide sequences after translation. LRRTM2-ECR coding sequence is attached to several peptide tag sequences (DYKDDDDK, TEV, Myc and His tags) and Biotin Acceptor Sequence (BAS). BirA is attached to DYKDDDDV tag. Secreted BirA biotinylates lysine of BAS sequence of LRRRTM2-ECR to bind to streptavidine beads.

DISCUSSION:

We developed novel method to examine presynapse formation stimulated with LRRTM2-beads using neuron ball culture. Currently, most of presynapse formation assay includes poly-D-lysine (PDL)-coated beads and dissociated culture/microfluidic chamber[20–22]. One of advantages of this method is LRRTM2-beads. While LRRTM2 interacts with neurexin to form excitatory presynapses specifically[11–13], PDL-beads induces both excitatory and inhibitory presynapses nonspecifically[20].

In this method, other presynapse organizers, whose members are over 10 proteins[3], are applicable to induce presynapses by changing extracellular domain of biotinylated protein depending on experimental purpose.

Another advantage is neuron ball culture. In some cases, conventional dissociated culture was used to analyze presynapse formation[20]. However, dissociated culture is not suitable to analyze low levels of synaptic proteins within presynapses, because overwhelming signals in cell bodies and dendrites interfere signals in presynapses. Instead, some groups use dissociated culture in microfluidic chamber that is special apparatus to culture axons and cell bodies separately in 2 comprtments[21,22]. Using microfluidic chamber, axonal sheet is formed in axonal compartment, and cell bodies are able to be removed from cell body compartment, similar to neuron ball culture. However, microfluidic chamber is special apparatus, and requires some skills to maintain constant culture condition. Neuron ball culture is not necessary to use special apparatus, and is relatively easy to be introduced as a new experimental method. Because essence of neuron ball culture is just to place neuronal cell aggregates (neuron balls) to glass-bottom dish/chamber, it can be easily combined with other methods. For example, it is considered that neuron ball culture using LRRTM2-beads is applicable for high content screening to measure fluorescence of 10-20 beads area at same time.

Critical step of this protocol is coating with PLL. If PLL coating is not uniform, axons of neuron balls would not extend uniformly in all direction. This disturbs efficient analysis of presynapse formation. We use glass coverslips and glass-bottom dishes, however, glass is sometimes not so clean for neuronal culture and uniform coating with PLL. In this protocol, at first, glass coverslips and glass-bottom dishes are soaked in neutral non-phosphorous detergent for 1-3 overnight, and then washed 8 times with ultrapure water. We use PLL whose molecular weight > 300,000 to reduce its concentration (15 μg/mL) for lower undesirable background. If lower molecular weight of PLL (*e.g.*, 30,000-70,000) is used, higher concentration (100 μg/mL) is necessary to extend axons.

Limitation of this method is that neuron ball culture cannot maintain > DIV15-16. Axons of neuron balls are fragmented after DIV15-16. Fragmented axons do not produce any presynapse after LRRTM2 beads application. Thus, this method is not applicable to analyze mature neurons (> DIV21). However, in most cases[11, 12, 21, 22], presynapse formation assay uses younger neurons that are cultured until DIV14. Another limitation is that axons without cell bodies cannot survive over 4 h. We analyzed accumulation of 5 synaptic proteins in presynapses so far, the accumulation of all proteins we checked reached almost plateau within 4 h (unpublished observation). It is considered that synaptic proteins accumulate enough at 4 h to analyze where synaptic proteins are derived from (cell body or axon).

Combination of neuron ball culture with LRRTM2-beads is relatively simple and flexible to adapt many experimental platforms. We have already applied this method to measure presynaptic activity using AM1-43 dye (unpublished observation). This method is considered to be applicable for high throughput screening. Presynapse formation assay is possible to apply high content screening by staining synaptic proteins in presynapses formed under LRRTM2-beads. This

method would contribute to find new compounds to cure neurological disease.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1.

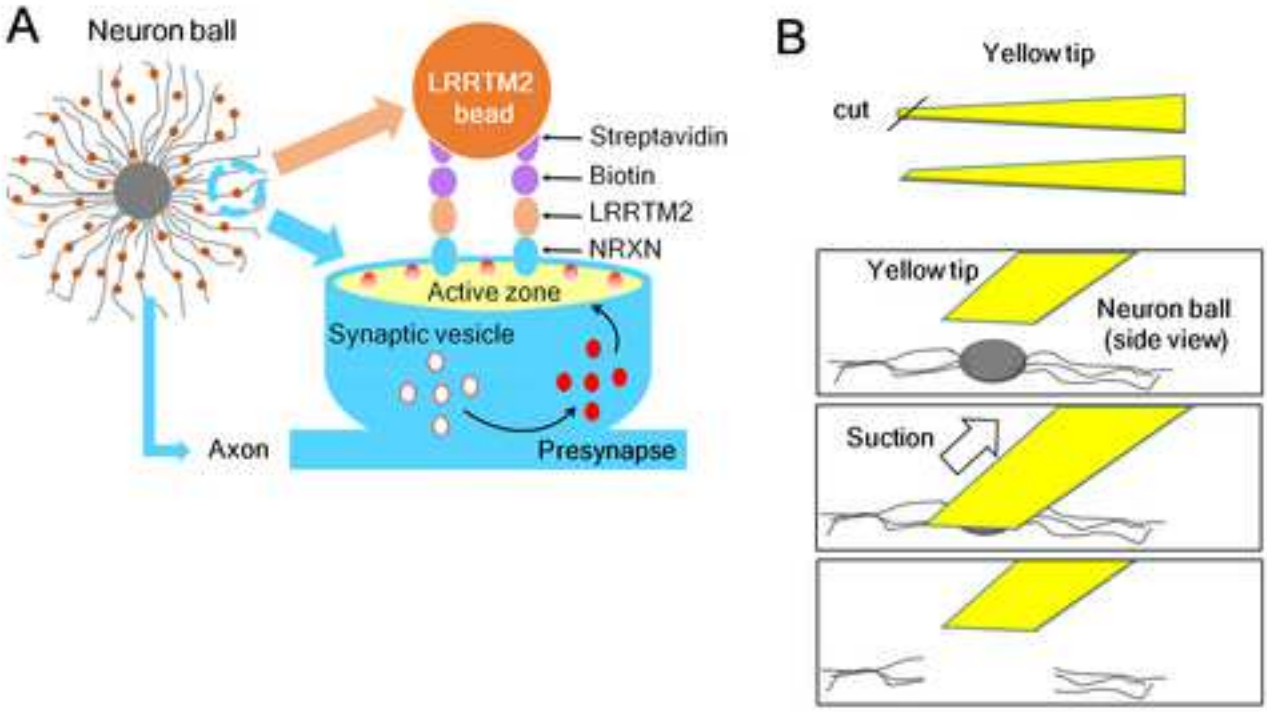


Figure 2.

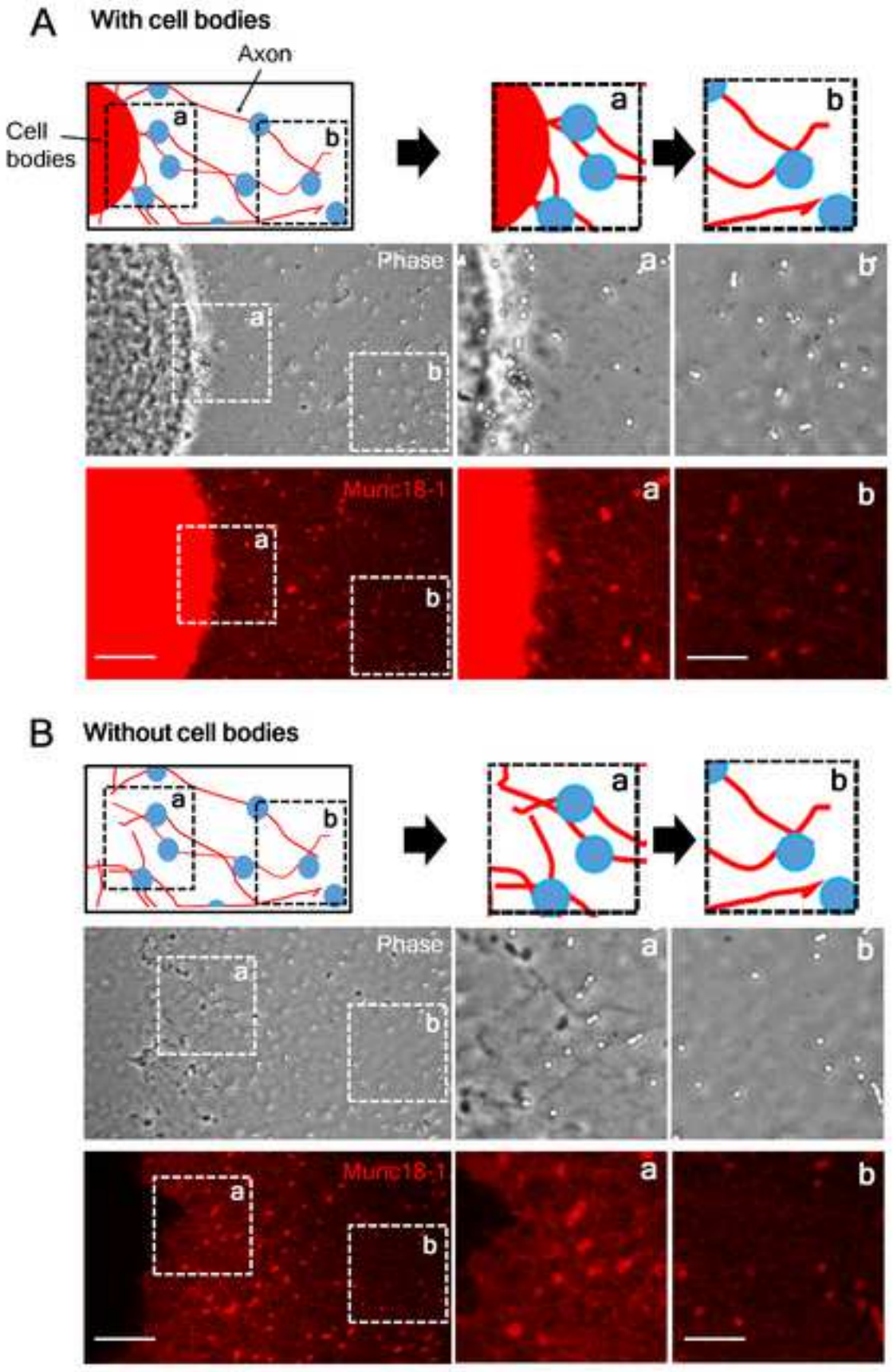


Figure 3.

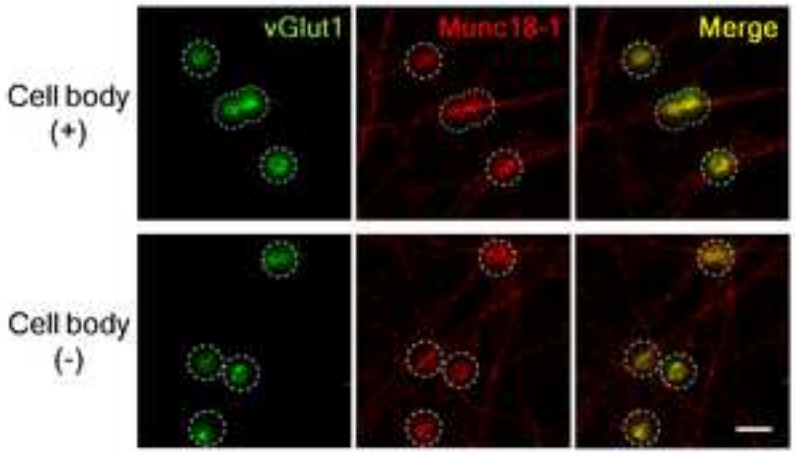
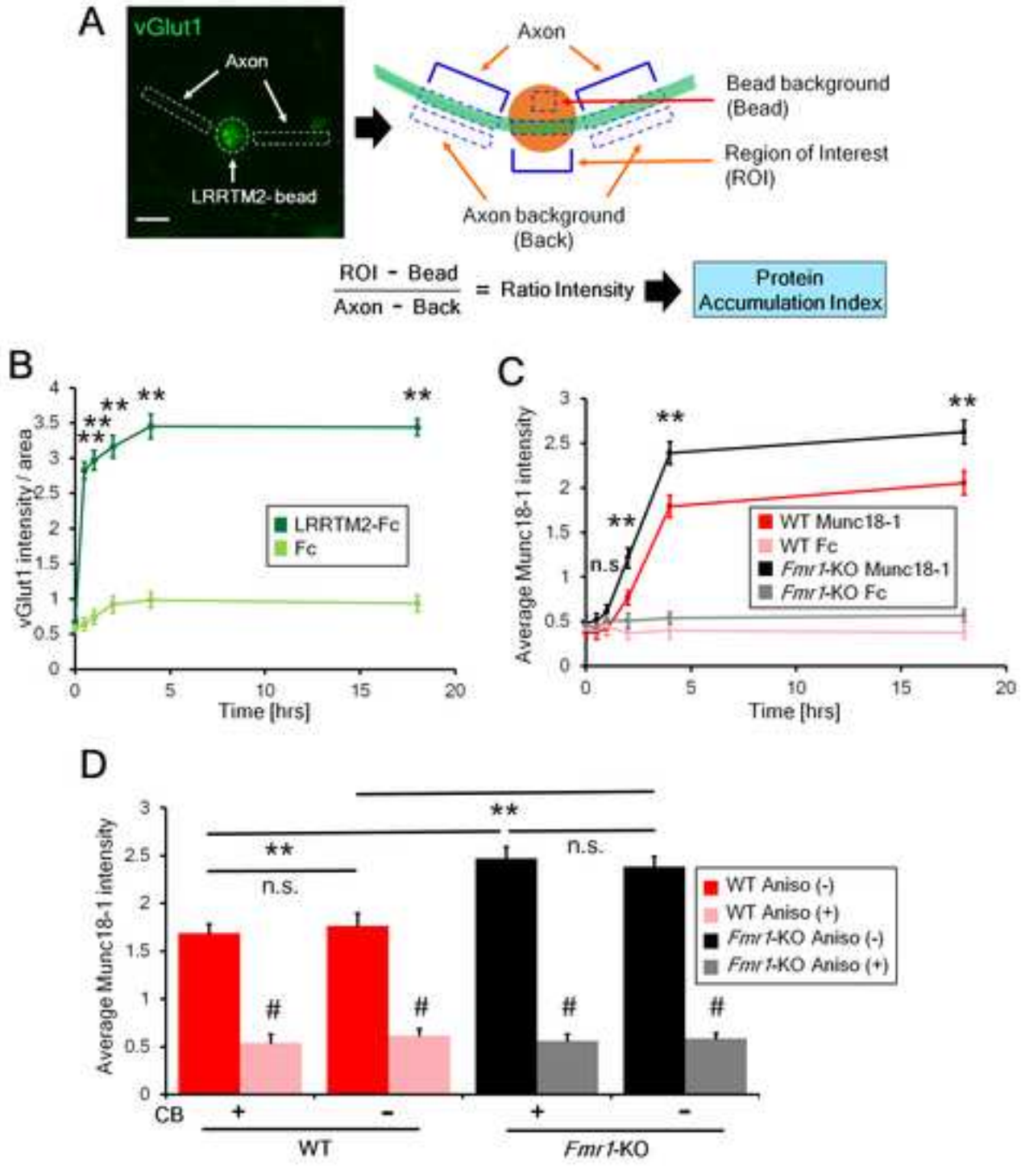


Figure 4.



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Name of Material/ Equipment	Product name	Company	Catalog Number	Comments/Description
	Antibody diluent	DAKO	S2022	
	Alexa Fluor 594 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson ImmunoResearch	715-585-151	
	Alexa Fluor 488 AffiniPure Donkey Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch	711-545-152	
	mouse anti-Munc18-1	BD Biosciences	610336	
	B-27 Supplement (50X), serum free	Thermo Fisher Scientific	17504044	
	Bovine Serum Alubumin (BSA)	Nacalai Tesque	01863-48	
	Cell-Culture Treated Multidishes (4 well dish)	Nunc	176740	
	Complete EDTA-free	Roche	11873580001	
	cooled CCD camera	Andor Technology	iXON3	
	Coverslip	Matsunami	C015001	Size: 15 mm, Thickness: 0.13-0.17 mm
	Cytosine β-D-arabinofuranoside (AraC)	Sigma-Aldrich	C1768	
	4',6-Diamidino-2-phenylindole Dihydrochloride (DAPI)	Nacalai Tesque	11034-56	
	Deoxyribonuclease 1 (DNase I)	Wako pure chemicals	047-26771	
	Expi293 Expression System	Thermo Fisher Scientific	A14635	
	Horse serum	Sigma-Aldrich	H1270	
	image acquisition software	Nikon	NIS-element AR	
	Image analysis software	NIH	Image J	https://imagej.nih.gov/ij/
	Inverted fluorecent microscope	Nikon	Eclipse Ti-E	
	GlutaMAX	Thermo Fisher Scientific	35050061	
	Neurobasal media	Thermo Fisher Scientific	#21103-049	
	Normal Goat Serum (NGS)	Thermo Fisher Scientific	#143-06561	
	N-propyl gallate	Nacalai Tesque	29303-92	
	Paraformaldehyde (PFA)	Nacalai Tesque	26126-25	
	Paraplast Plus	Sigma-Aldrich	P3558	
	Poly-L-lysine Hydrobromide (MW > 300,000)	Nacalai Tesque	28359-54	
	poly (vinyl alcohol)	Sigma	P8136	
	Prepacked Disposable PD-10 Columns	GE healthcare	17085101	
	rabbit anti-vesicular glutamate transporter 1	Synaptic Systems	135-302	
	SCAT 20X-N (neutral non-phosphorous detergent)	Nacalai Tesque	41506-04	
	Streptavidin-coated magnetic particles	Spherotech Inc	SVM-40-10	diameter: 4-5 µm
	TritonX-100	Nacalai Tesque	35501-15	
	Trypsin	Nacalai Tesque	18172-94	

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Author(s):	Shumaia Parvin, Renoma Takeda, Yukio Sasaki

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April 5th, 2019

Journal of Visualized Experiments (JoVE)

Dear Editors,

Thank you very much for your mail of March 19th concerning our manuscript (Ref: JoVE59893) entitled "Presynapse formation assay using presynapse organizer beads and “Neuron ball” culture" together with the reviewers’ comments. We are pleased to learn that you are interested in our paper for publication in Journal of Visualized Experiments (JoVE).

We have carefully examined the editorial and reviewers’ comments and will now respond to each of the comments in turn, indicating revisions made to the text as appropriate.

- **Editorial comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have checked proofread our manuscript thoroughly.

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We have used 12 pt Calibri font. We have changed single-spaced text throughout the manuscript.

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We checked the numbering of the protocol.

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We added a one-line space between protocol steps.

6. JoVE cannot publish manuscripts containing commercial language. This includes company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

We have removed company names from our protocol. For example, We changed "MilliQ water" and "SCAT 20X-N" to "ultrapure water" and neutral non-phosphorous detergent, respectively.

7. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc.

We changed "ml" to mL"

8. Please use h, min, s for time units.

We changed "hr" to "h".

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We highlight essential steps in yellow within 2.75 pages.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The MS reported by Parvin et al. describes the sophisticated method that makes possible to observe synapse formation easily in vitro. The protocol is well explained.

We thank reviewer #1 for highly appreciating our protocol.

Minor Concerns:

The only concern is, (although it is not directly related to the method) the lack of the sequence and vector information of LRRTM2 expression system. To reproduce the experiment, it is necessary to be described in detail.

We have added the vector information as a supplementary figure 1. We also added figure legend for supplementary figure 1 in FIGURE AND TABLE LEGENDS section.

Reviewer #2:

Manuscript Summary:

The authors describe a method for studying presynapse formation by combining pseudo-explant or neuron ball cultures with the axonal application of LRRTM2 coated beads. The methods allows for the removal of the cell bodies and the study of local protein synthesis without the use of microfluidic chambers.

We also thank reviewer 2 for highly appreciating our protocol.

Major Concerns:

p.4 line 89: most of the solutions should not be autoclaved as they contain serum, glucose, etc.

Major copy-editing is needed.

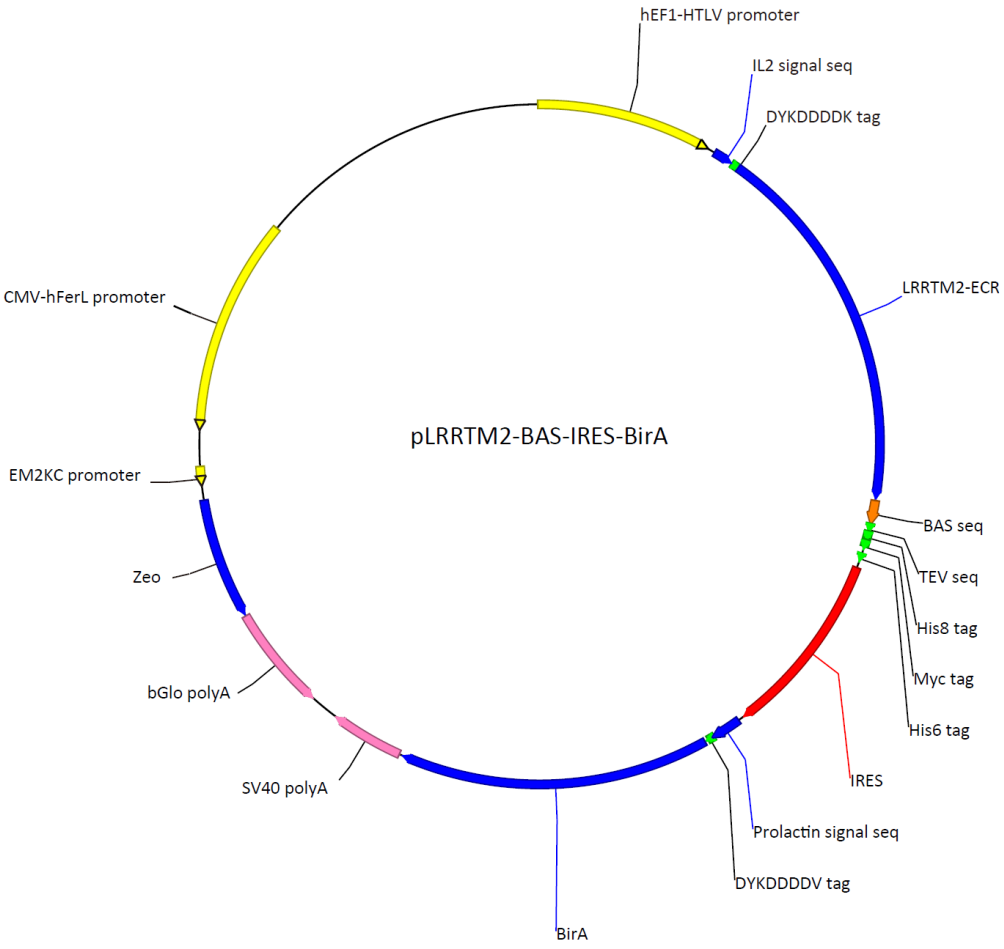
We understand autoclave is not allowed to sterilize some solutions, because serum and some reagent is vulnerable to high temperature. For heat vulnerable solution, we used filtration for sterilization. We changed the old version of sentence “Prepare and autoclave all the required solutions in advance” to “Prepare all the required solutions and sterilize them by autoclave/filtration in advance” (line 89).

We also checked spelling and typo errors in our manuscript. We hope that you will find these changes satisfactory and the revised version is now acceptable for publication in *JoVE*. We wish to thank you again for your thorough and prompt review of our manuscript and to thank the reviewers for their constructive and useful criticisms and suggestions.

Sincerely,

Yukio Sasaki, Ph.D.
Associate professor
Department of Medical Life Science
Yokohama City University Graduate School of Medical Life Science
Yokohama 230-0045, Japan.

Supplementary Fig. 1



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