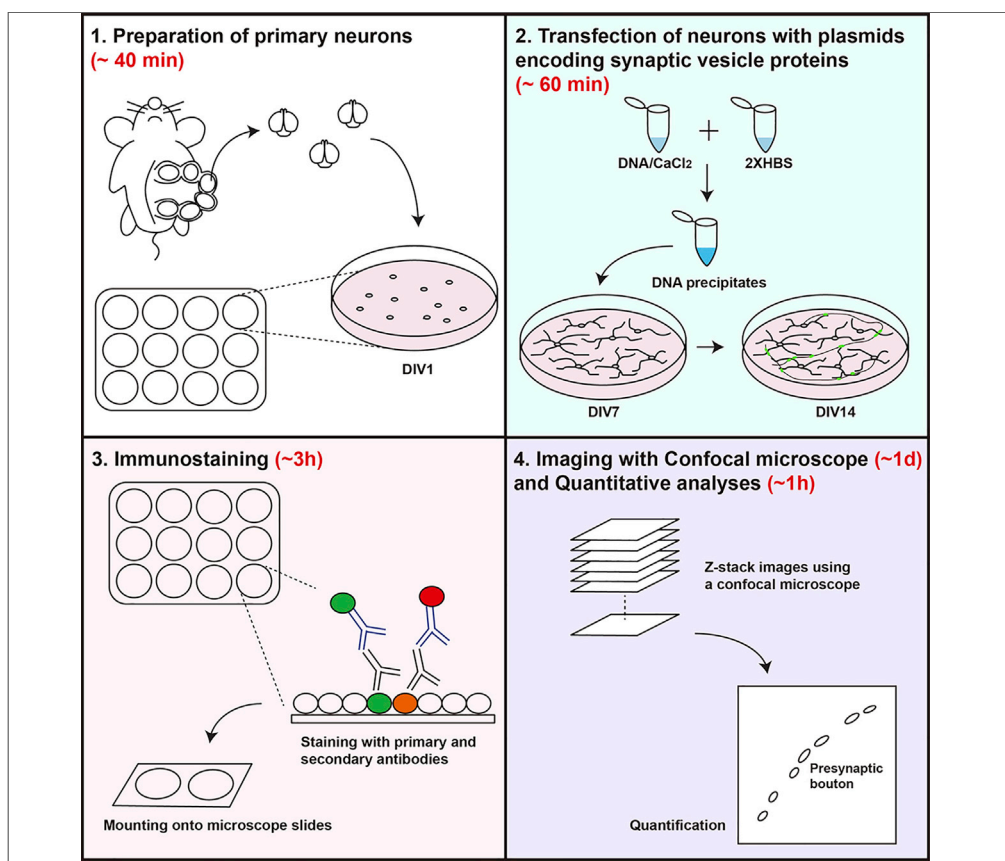


## Protocol

# Protocol for Quantitative Analysis of Synaptic Vesicle Clustering in Axons of Cultured Neurons



Clustering of synaptic vesicles along the neuronal axons is a critical mechanism underpinning proper synaptic transmission. Here, we provide a detailed protocol for analyzing the distribution of synaptic vesicles in presynaptic boutons of cultured neurons. The protocol covers preparation of cultured neurons, expression of synaptic vesicle-enriched proteins, and quantification procedures. Utilizing neurons from postnatal transgenic mice, this method can be applied to investigate the roles of synaptic genes in regulating vesicle dynamics at synaptic sites.

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### HIGHLIGHTS

Detailed methods for preparation of cultured neurons and transfection of plasmid DNA

Detailed guidelines for analyzing synaptic clustering in axons of presynaptic neurons

Description and troubleshooting of the most common pitfalls associated with the procedure

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## Protocol

## Protocol for Quantitative Analysis of Synaptic Vesicle Clustering in Axons of Cultured Neurons

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<https://doi.org/10.1016/j.xpro.2020.100095>

## SUMMARY

Clustering of synaptic vesicles along the neuronal axons is a critical mechanism underpinning proper synaptic transmission. Here, we provide a detailed protocol for analyzing the distribution of synaptic vesicles in presynaptic boutons of cultured neurons. The protocol covers preparation of cultured neurons, expression of synaptic vesicle-enriched proteins, and quantification procedures. Utilizing neurons from postnatal transgenic mice, this method can be applied to investigate the roles of synaptic genes in regulating vesicle dynamics at synaptic sites.

For complete details on the use and execution of this protocol, please refer to Han et al. (2020a).

## BEFORE YOU BEGIN

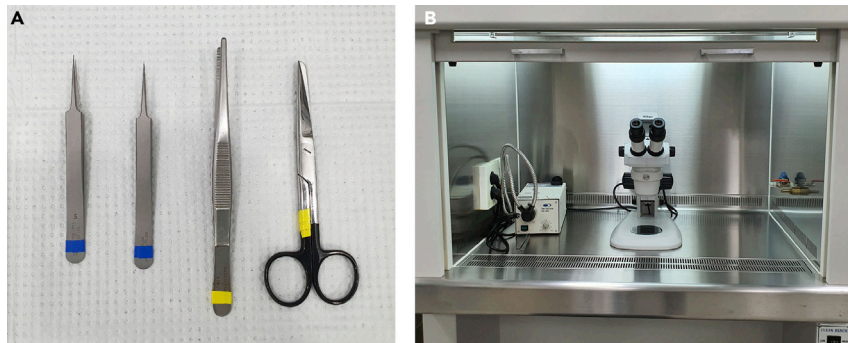
This protocol describes the reagents, equipment, and experimental steps required for preparing dissociated primary cultured neurons and quantitatively analyzing synaptic vesicle clustering, distribution, and/or localization in presynaptic axons. We have performed these procedures for more than a decade, but have never published a full detailed protocol as a separate article. Unless otherwise stated, we describe preparation of rat cultured neurons and concomitant analyses, although we would note that this protocol is equally applicable to the preparation of cultured mouse neurons. C57BL/6 mice (2–8 months old) were used as breeders for the purposes of obtaining pregnant female mice. Successful mating can be confirmed by detection of a vaginal plug in the female, by palpitation, or by visual confirmation of pregnancy. Coated coverslips and various media should be prepared in advance (i.e., the day before preparation of neuron cultures). For preparation of neuron culture media, please refer to “Materials and Equipment”.

## Prepare Coated Coverslips

⌚ Timing: 2 days

1. Incubate coverslips in concentrated nitric acid in ceramic racks for at least 24 h at 24°C–26°C.
2. Discard the nitric acid in a chemical fume hood in accordance with institutional guidelines.
3. Rinse coverslips in a Petri dish by incubating with deionized water for 30 min, then discard the rinse solution.





**Figure 1. Preparation of Surgical Instruments and Dissection Hood**

(A) Composition of surgical packs.

(B) Dissection hood equipped with a microscope.

4. Repeat Step 3 five times.
5. Sterilize coverslips in a muffle furnace (JEIO Tech; Cat# MF-G) at 240°C for 4 h.
6. Transfer coverslips to a sterilized Petri dish in a culture hood and store them at 24°C–26°C.
  - a. Prepared coverslips can be stored in a culture hood after exposure to ultraviolet light for 30 min and used for up to ~1 month.
  - b. Perform steps 7 and 8 one day prior to dissection procedures.
7. Place a sterilized coverslip in each well of a 12-well plate.
8. Add 1 mL of 0.1 mg/mL poly-D-lysine in 0.1 M boric acid (pH 8.5) to wells containing coverslips, and allow plates to sit for 12–16 h at 37°C in a CO<sub>2</sub> incubator.
9. After 12–16 h, wash the coverslips with autoclaved deionized water on a clean bench.

**Note:** It is critical to prevent coverslips from drying before use.

### Preparation of Surgical Instruments

⌚ Timing: 5 min

10. Unfold autoclaved surgical packs (Figure 1A).
11. Sterilize forceps and scissors with 70% ethanol.
12. Turn on the dissection hood and clean the inside with 70% ethanol (Figure 1B).

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
Bassoon	Enzo life Sciences	Cat # SAP7F407; RRID: AB_2313990
Cy3-conjugated donkey anti-mouse	Jackson ImmunoResearch laboratories	Cat #715-165-150; RRID: AB_2340813
FITC-conjugated donkey anti-goat	Jackson ImmunoResearch laboratories	Cat #705-095-147; RRID: AB_2340401
EGFP	Rockland	Cat # 600-101-215; RRID: AB_218182
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
Neurobasal medium	ThermoFisher Scientific	Cat #21103049
B-27 supplement (50×)	ThermoFisher Scientific	Cat #17504-044

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Penicillin/streptomycin	ThermoFisher Scientific	Cat #15140122
HBSS (Hank's Balanced Salt Solution)	ThermoFisher Scientific	Cat #14065056
Sodium pyruvate	ThermoFisher Scientific	Cat #11360070
GlutaMax supplement	ThermoFisher Scientific	Cat #35050061
Minimal essential medium (MEM)	Welgene	LM007-12
Poly-D-lysine hydrobromide	Sigma-Aldrich	P0899
37% formaldehyde	Biosesang	F1012
Bovine serum albumin	Sigma-Aldrich	A7906
Horse serum	Gibco	16050122
EDTA	Affymetrix	15701
Calcium chloride	Affymetrix	12531
Magnesium chloride	Sigma-Aldrich	M8266
Papain	Worthington	LS003126
Hydrochloric acid	Reagents Duksan	Cat #1129
Sodium hydroxide	DAEJUNG	7572-3700
Sucrose	Sigma-Aldrich	S7903
Triton X-100	Sigma-Aldrich	T8787
Nitric acid	Reagents Duksan	Cat #1538
Boric acid	Sigma-Aldrich	B6768
<b>Critical Commercial Assays</b>		
NucleoBond® Xtra Midi kit for transfection-grade plasmid DNA	MACHEREY-NAGEL	MN740410
CalPhos Kit for neuron transfections	Takara	Cat #631312
<b>Experimental Models: Organisms/Strains</b>		
Mice: E17 pregnant females (C57BL/6J)	The Jackson Laboratory	000664
Rat: E18 pregnant females (Sprague-Dawley)	Daehan Biolink	N/A
<b>Recombinant DNA</b>		
pCAGG-VGLUT1-Venus	<a href="#">Han et al., 2020a</a>	N/A
<b>Software and Algorithms</b>		
MetaMorph	Molecular Devices	<a href="https://www.moleculardevices.com">https://www.moleculardevices.com</a>
Photoshop	Adobe	<a href="https://www.adobe.com">https://www.adobe.com</a>
Microsoft Excel	Microsoft	<a href="https://www.microsoft.com">https://www.microsoft.com</a>
Zen 2.6	Zeiss	<a href="https://www.zeiss.com/microscopy/int/products/microscope-software/zen-lite.html">https://www.zeiss.com/microscopy/int/products/microscope-software/zen-lite.html</a>
<b>Other</b>		
Surgical scissors	Fine Science Tools	Cat #14054-13
Student Dumont #5 forceps	Fine Science Tools	Cat #91150-20
Dressing forceps	KASCO	Cat #6-004
Dry ice	N/A	N/A
CO <sub>2</sub> incubator	ESCO	Cat # CCL-170B-8-FD
Centrifuge	Labogene	<a href="https://labogene.co.kr">https://labogene.co.kr</a>
Cover glasses	Marienfeld	Cat #0111580
0.2-µm syringe filters	Sartorius Stedim Biotech	<a href="https://www.sartorius.com">https://www.sartorius.com</a>

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dissecting microscope	Nikon	<a href="https://www.microscope.healthcare.nikon.com">https://www.microscope.healthcare.nikon.com</a>
Dissection hood	Bio-free	<a href="https://biofree.co.kr">https://biofree.co.kr</a>
Conical tubes	Neurex	<a href="https://saehanlab.co.kr">https://saehanlab.co.kr</a>
Microcentrifuge tubes	Axygen	<a href="https://www.coming.com">https://www.coming.com</a>
12-well plates, 6-well plates	SPL	<a href="https://www.spllifesciences.com">https://www.spllifesciences.com</a>
Furnace	JEIO Tech	Cat #MF-G
Petri dishes	SPL	<a href="https://www.spllifesciences.com">https://www.spllifesciences.com</a>
Confocal microscope (LSM800)	Zeiss	<a href="http://www.zeiss.com">http://www.zeiss.com</a>

**MATERIALS AND EQUIPMENT**

**Prepare Neuron Culture Media before Starting the Procedure**

**Complete Neurobasal (CNB) Media**

Reagent	Final Concentration	Volume (mL)
Neurobasal Medium	1×	472.5
B-27 Supplement	2%	10
Sodium pyruvate	1 mM	5
GlutaMax	1×	5
Penicillin Streptomycin	50,000 U/mL	5
Fetal Bovine Serum	0.5%	2.5
Total		500

**Papain Solution**

Reagent	Final Concentration	Volume (μL)
HBSS	1×	4,910
Papain	14 U/mL	80
0.5M EDTA	0.5 μM	5
1M CaCl <sub>2</sub>	1 μM	5
Total		5,000

**Note:** After mixing all reagents for the papain solution, vortex and filter the solution using a 0.2-μm syringe filter.

**Fixing Solution**

Reagent	Final Concentration	Amount
37% formaldehyde	3.7%	50 mL
10× PBS	1×	50 mL
Sucrose	4%	20 g
Deionized water	n/a	400 mL
Total		500 mL

### Permeabilization Solution

Reagent	Final Concentration	Volume (mL)
10 % Triton X-100	0.2%	4
10× PBS	1×	20
Deionized water	n/a	176
Total		200

### PBS-BH Solution

Reagent	Final Concentration	Amount
10× PBS	1×	50 mL
Bovine serum albumin	0.1%	0.5 g
Horse serum	3%	15 mL
Deionized water	n/a	435 mL
Total		500 mL

**Note:** After mixing all reagents for the phosphate-buffered saline–bovine serum albumin/horse serum (PBS-BH) solution, filter the solution using a 0.2- $\mu$ m syringe filter.

### Poly-D-lysine Solution

Reagent	Final Concentration	Volume (mL)
2 mg/mL Poly-D-lysine	0.1 mg/mL	2.5
0.1 M Boric acid	0.1 M	47.5
Total		50 mL

## STEP-BY-STEP METHOD DETAILS

### Tissue Harvesting

⌚ Timing: 20 min

1. When using late embryonic stage (E17–18) rat fetuses, euthanize the dam, then remove the uterus and free individual fetuses from the embryonic sack (Figure 2).
2. Place fetuses into a sterile Petri dish and continue as outlined below.
3. Decapitate rat fetus or pup, following guidelines approved by the researcher's Institutional Animal Care and Use Committee.
4. Remove skin and skull and place each brain in a 60-mm Petri dish containing ice-cold Hank's Balanced Salt Solution (HBSS) onto an ice pack.
5. Under the dissecting microscope, dissect hippocampi or cortices one at a time in a 100-mm Petri dish containing enough dissection solution to cover the tissue.
6. Once meninges are removed and the hippocampus or cortex is freed from the brain, cut out the extra tissue and transfer the hippocampus or cortex to a 35-mm Petri dish containing HBSS.

### Tissue Dissociation

⌚ Timing: 20 min

7. After all hippocampi or cortices have been collected, transfer them to a 15-mL conical tube containing papain solution and allow them to incubate at 37°C for 15 min.



**Figure 2. Preparation of Rat Fetuses for Primary Neuron Culture**

- (A) Euthanized pregnant female rat.  
 (B) Opening in the mid-ventral side of the rat.  
 (C) Prenatal pups in the uterus.  
 (D) Opening the uterus and removing pups using autoclaved sterile forceps.

**Note:** Prepare papain solution freshly in a 15-mL conical tube immediately before starting dissection.

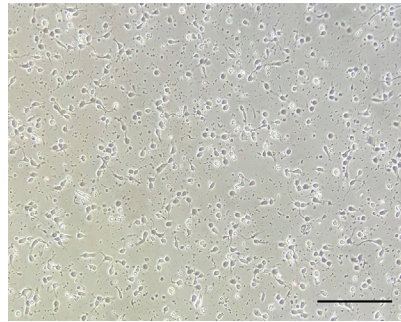
**Note:** During the papain incubation step, wash the coverslips three times with deionized water, place in wells of a 12-well plate, and add 1.5 mL warmed CNB medium to each well.

8. Stop the digestion by removing papain solution using a 1-mL pipette.
9. Add 5 mL of HBSS, close the tube and invert it gently a few times. Let the hippocampi or cortices settle for about 2 min and then remove the HBSS.
10. Repeat Step 9 two more times.
11. Add 1 mL of CNB medium and triturate tissues with a fire-polished Pasteur pipette 40 times. Try not to introduce air bubbles during this step.

### Plating Dissociated Cultured Neurons on Coated Coverslips

⌚ Timing: 5 min

12. Dilute 20  $\mu$ L of cell suspension into 180  $\mu$ L of plating media and count cells on a hemocytometer.
13. Plate  $3.0 \times 10^5$  cells onto 18-mm poly-D-lysine-coated coverslips placed in wells of a 12-well plate containing warmed CNB medium.
14. Maintain cultures at 37°C and 5% CO<sub>2</sub>.



**Figure 3. Micrograph of Primary Hippocampal Neurons at 1 Day In Vitro (DIV1)**  
Scale bar, 0.2 mm.

- The next day, replace the medium with fresh CNB medium (Figure 3).

**Note:** It is important to monitor cultures daily for potential problem; otherwise, planned experiments could be compromised. However, observations should be as brief as possible to minimize stress (e.g., light exposure, pH and temperature variations) on the cells.

### Transfection of Cultured Neurons with Plasmids

⌚ **Timing:** 60 min

- At about DIV7, check the quality of the plated neurons by observing them under a microscope (Figure 4).
- For each transfection, prepare Solution A and Solution B in separate sterile microcentrifuge tubes according to the manufacturer's instructions (CalPhos Mammalian Transfection Kit). For each  $\sim 3.5 \times 10^5$  neurons plated in a 12-well plate, make Solution A by mixing 8  $\mu\text{g}$  of pCAGG-VGLUT1-Venus plasmid DNA with 9  $\mu\text{L}$  of a 2 M  $\text{CaCl}_2$  solution and sufficient deionized water to bring the total volume to 75  $\mu\text{L}$ . Solution B consists of 75  $\mu\text{L}$  of 2 $\times$  HBS.

**Note:** Prepare pCAGG-VGLUT1-Venus plasmids by DIV7 using an endotoxin-free DNA Midi Kit.

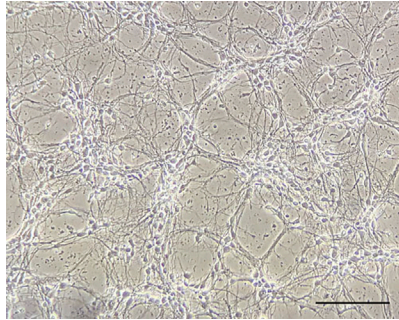
- Carefully vortex Solution A while adding Solution B dropwise (usually takes 30 s~1 min per reaction).
- Incubate the mixed A+B Solution at 24°C–26°C for 30 min.
- While the A+B solution incubates, use forceps to transfer a coverslip with plated neurons to another 12-well plate containing 2 mL of MEM and 10  $\mu\text{L}$  of 1 M  $\text{MgCl}_2$ .

**Note:** Retain the 12-well plate containing CNB medium conditioned by the original cultures of coverslip-plated neurons.

- Gently vortex transfection (A+B) solution and then add the solution dropwise to wells of a 12-well plate containing coverslip-plated neurons.
- Gently move the plate back and forth to evenly distribute the transfection solution.
- Incubate the plate at 37°C in a 5%  $\text{CO}_2$  incubator for 15 min.

**⚠ CRITICAL:** it is imperative that precipitates can be clearly observed under a microscope after the 15-min incubation period. If precipitates are not detected, incubate for an additional 5–10 min.





**Figure 4. Micrograph Showing the Morphology of Primary Hippocampal Neurons at DIV7**

Scale bar, 0.2 mm.

24. Prepare 6-well plates containing 2 mL of HBSS per well (three wells are normally needed to process each coverslip).
25. Transfer the coverslip to the first well of a 6-well plate containing 2 mL of HBSS and gently shake the plate.
26. Transfer the coverslip to the second well of a 6-well plate containing 2 mL of HBSS and gently shake the plate.
27. Transfer the coverslip to the third well of a 6-well plate containing 2 mL of HBSS and gently shake the plate.
28. Transfer the coverslip to the 12-well plate containing the original neuron-conditioned CNB medium.
29. Twenty-four hours later, assess the success of transfection by monitoring VGLUT1-Venus fluorescence in neurons under a fluorescence microscope.

⚠ **CRITICAL:** Transfected neurons start to become visible under a fluorescence microscope ~12 h after transfection.

### Immunocytochemistry

⌚ **Timing:** ~3 h

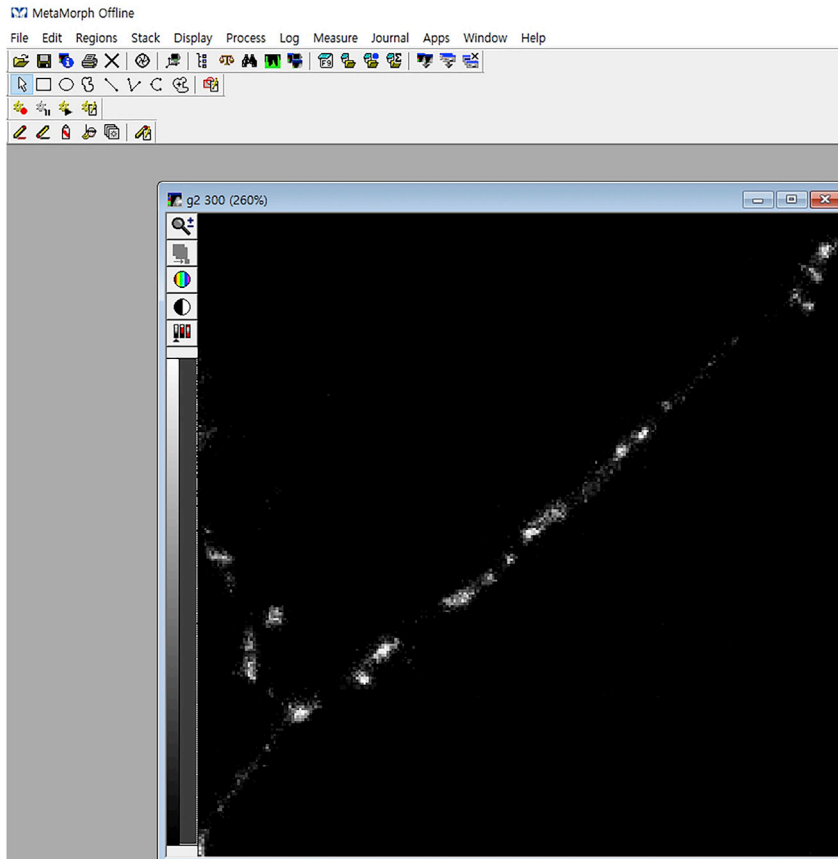
30. At DIV14, aspirate CNB medium from 12-well plates and wash coverslip-plated neurons by adding ice-cold PBS.

**Note:** Before immunocytochemistry procedures, inspect all transfected neurons under a fluorescence microscope to ensure their viability and health.

31. Add fixing solution and incubate for 10 min at 4°C.
32. Aspirate the fixing solution and wash neurons by adding ice-cold PBS.
33. Repeat Step 32 twice more.
34. Add permeabilization solution to 12-well plates and incubate at 24°C–26°C for 10 min.
35. Aspirate the permeabilization solution and incubate with PBS-BH solution at 24°C–26°C for 15 min to block nonspecific binding of antibodies.
36. Incubate with primary antibodies as follows:
  - a. Prepare 100  $\mu$ L of primary antibody solution (anti-Bassoon [diluted 1:100] and anti-GFP [diluted in 1:1,000] in PBS-BH solution) per coverslip.

**Note:** Antibodies that label other active zone proteins (e.g., Piccolo, RIM1, or Munc13-1) would serve the same purpose as the anti-Bassoon antibody and could be substituted for it in this step.





**Figure 6. An Opened Gray Image in MetaMorph Software**

### Confocal Imaging

⌚ Timing: 1–3 days (depending on the number of coverslips)

45. Acquire Z-stack images in standard mode using a laser-scanning confocal microscope (Zeiss LSM800 with Airyscan module) equipped with a 63× objective (0.156 μm/pixel; X-Y dimension 1,024 × 1,024) and Zen2.6 Software (Zeiss).
46. Configure Z-stack images using Acquisition Dimensions and four slices with a 1.2-μm interval setting.
47. Generate maximum intensity projections for all captured Z-stack files (Figure 5).
48. Save max intensity projection files in 16-bit Tiff file format.

### Quantitative Analyses

⌚ Timing: 1 h

49. Separate RGB colors in images using Photoshop (Adobe) and save each image in gray mode in-Tiff file format.
50. Open gray image files corresponding to transfected neurons expressing VGLUT1-mVenus into MetaMorph software (Molecular Devices) (Figure 6).
51. Calibrate pixel length in images using the Length Calibration Function (Figure 7).

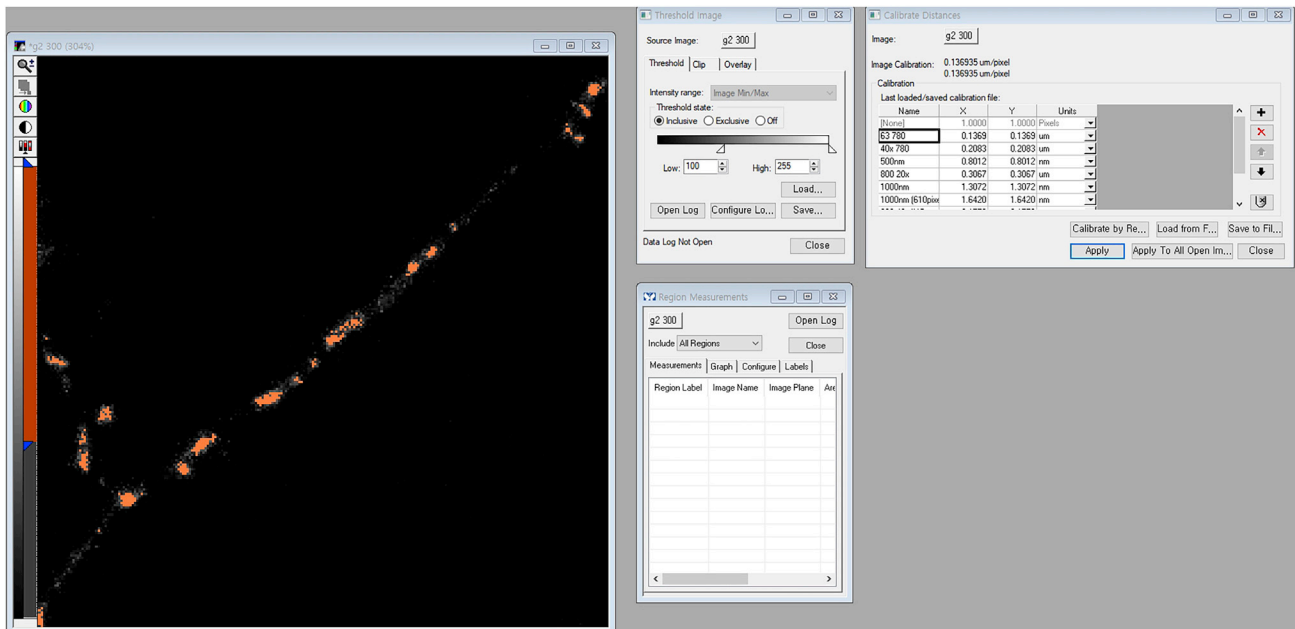
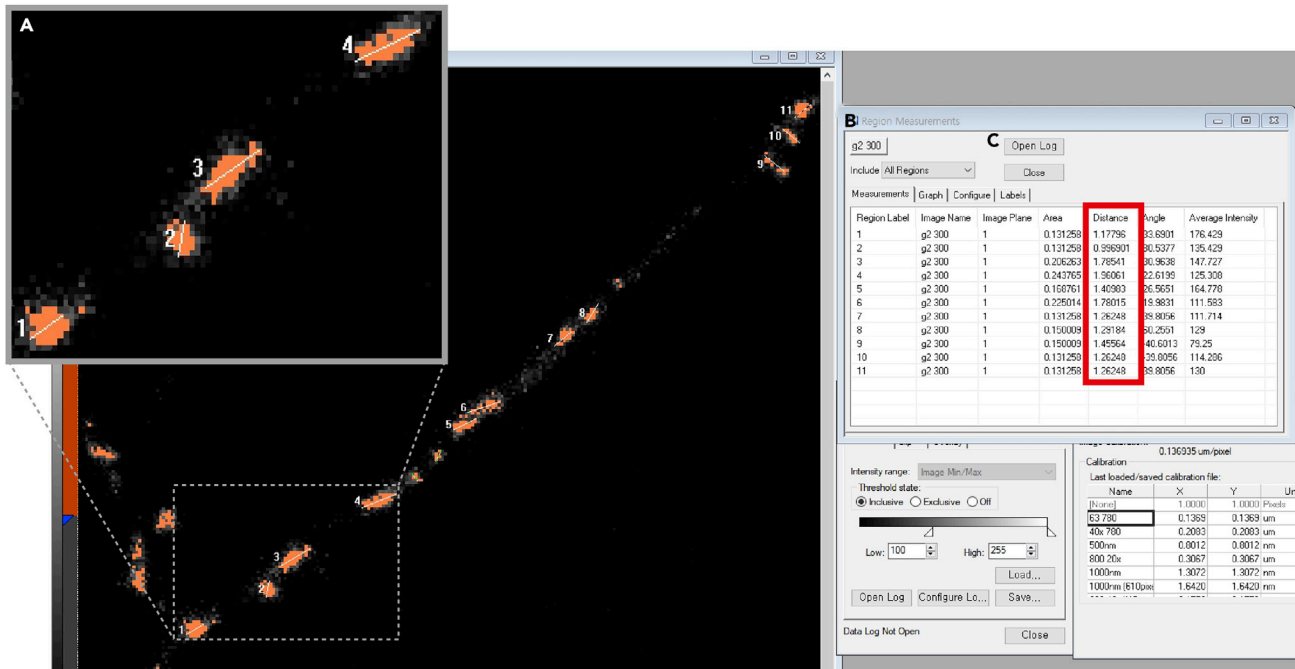


Figure 7. Calibrating Distances and Thresholding a Gray Image in MetaMorph Software

52. To remove background signals, set the threshold using the Threshold Function (minimum, 100; maximum, 255) such that pixels above the minimum threshold take on an orange coloration (Figure 7).
53. Measure presynaptic bouton length as follows:
  - a. Draw a straight line across each orange region (Figure 8A).
  - b. Open a Region Measurement window (Figure 8B).
  - c. Open Log to link to Microsoft Excel (Figure 8C).
  - d. Click Log Data to export data to a Microsoft Excel sheet. The values of all parameters of the Region Measurement window are exported to the Excel sheet. The average of Distance values corresponds to the length of a presynaptic bouton (Figure 8; red box).
54. Determine the percentage of VGLUT1-positive Bassoon puncta as follows:
  - a. Draw a line surrounding an axonal bouton labeled with VGLUT1-Venus to define the region of interest (ROI) (Figure 9A).
  - b. Save each ROI in Rgn file format (Figure 9B).
  - c. Open gray images files corresponding to Bassoon puncta in MetaMorph software.
  - d. Calibrate pixel length in images using the Length Calibration Function (Figure 10A).
  - e. To remove background signals, set the threshold using Threshold Function (minimum, 100; maximum, 255) such that pixels above the minimum threshold take on an orange coloration (Figure 10B).
  - f. Load saved ROIs for the gray image corresponding to Bassoon puncta (Figures 10C and 11A).
  - g. Open a Region Measurement window (Figure 11B)
  - h. Click Open Log to link to Microsoft Excel (Figure 11C).
  - i. Click Log Data to export data to an Excel sheet. The values of all parameters of the Region Measurement window are exported to the Excel sheet. Average intensity indicates the intensity of VGLUT1-positive Bassoon (Figure 11; red box).

**Note:** Instead of using the commercial software packages described in this protocol (i.e., MetaMorph, Photoshop and Microsoft Excel), free, open-source programs with similar functions could be used (e.g., ImageJ, GNU Image Manipulation Program [GIMP], and LibreOffice).



**Figure 8. Measurement of Presynaptic Bouton Length in MetaMorph Software**  
(A) Drawing a line across each presynaptic bouton.  
(B and C) Measuring the bouton length and exporting it to a Microsoft Excel sheet.

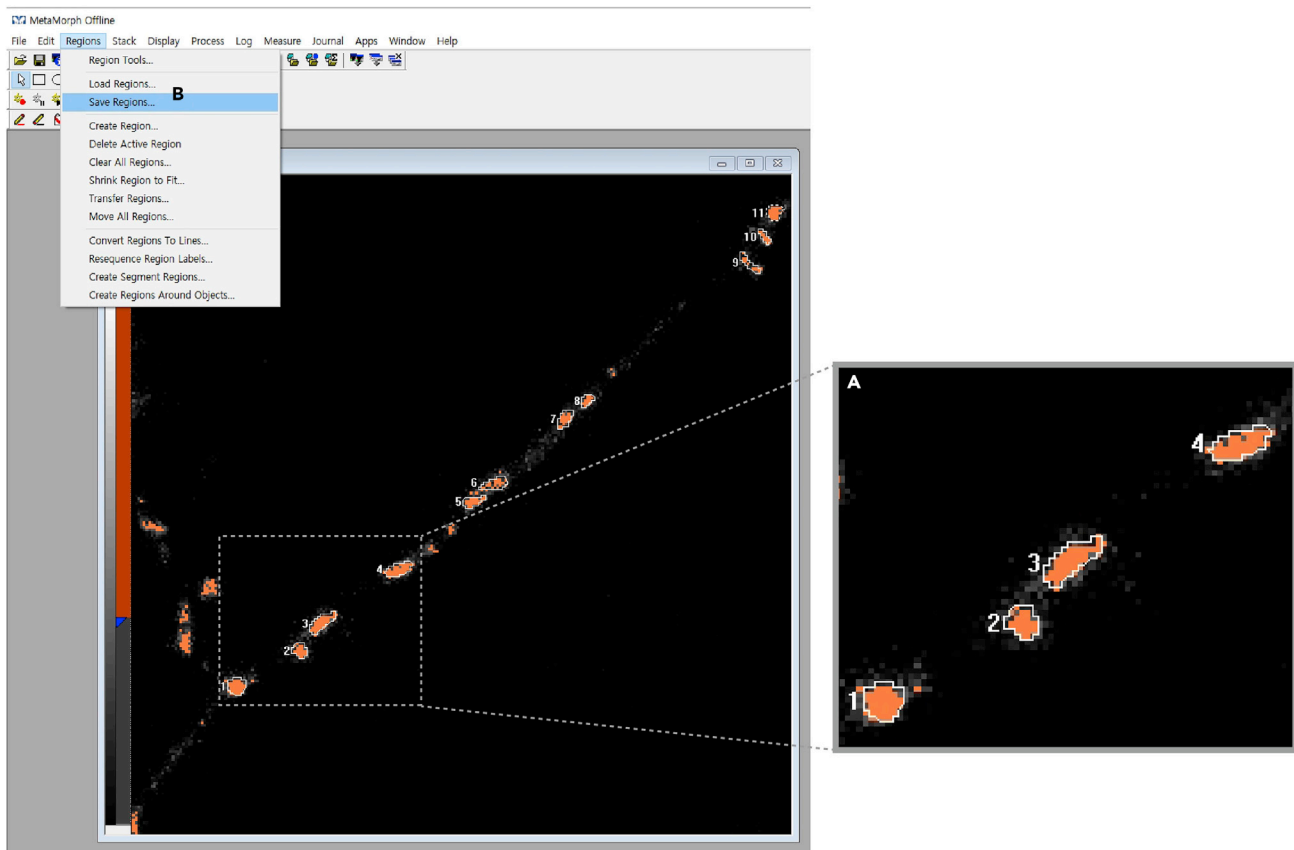
## EXPECTED OUTCOMES

A non-stressed female normally has 8–12 embryos. Embryos that are too small or too large will result in a decrease in the survival rate of plated cells within the first few days after culturing. Acid-washed coverslips, neuron culture media, and plasmid DNAs should be prepared in advance (i.e., at least 1 day before) based on precise information about mating date.

In a typical imaging experiment, cultures should be plated at a density of  $3.5 \times 10^5$  cells per 12-well plate. Obtaining high-quality cultures is highly skill-dependent, but is easily achievable with care and practice. Several points should be carefully considered prior to culturing ([Troubleshooting 1](#)).

Cultured neurons can be infected with recombinant lentiviruses expressing the gene of interest. In our recent studies ([Han et al., 2020a](#); [Han et al., 2020b](#)), we produced lentiviruses expressing Cre recombinase (using inactive Cre recombinase as a negative control) and used them to infect cultured (~DIV3–4) neurons derived from mice harboring a floxed allele of the targeted presynaptic adhesion molecules. Lentiviruses are produced in HEK293T cells by transfections of the lentivirus backbone vectors, psPAX2 and pMD2.G, followed 72 h later by replacement of the supernatant with neuron culture media (i.e., complete neurobasal media). In our experience, a 7-day incubation of cultured neurons with lentiviral supernatant works well in most cases (>95% infection rate), although we observe a sharp decrease in virus titer for lentiviral vectors with large inserts (>8 kb).

Cultured neurons can also be transfected with any mammalian expression vector(s) using the  $\text{Ca}^{2+}$ -phosphate method. Successful transfections using this method rely on experience with pipetting to generate precipitates. Note that, in our hands, lentiviral infections work well using DIV2–6 cultured neurons, whereas plasmid transfections work well using DIV3–14 cultured neurons. If the lentiviral vector or the expression plasmid harbors fluorophore moiety, neurons expressing exogenously introduced protein can be easily recognized using fluorescence microscopy, which, in our case, is set up in the tissue culture room.



**Figure 9. Drawing Lines Defining ROIs Corresponding to VGLUT1-Venus-Positive Puncta**  
(A) Drawing a line surrounding a presynaptic bouton visualized by VGLUT1-Venus fluorescence.  
(B) Saving each ROI in Rgn file.

Toxicity resulting from lentiviral infections and/or  $\text{Ca}^{2+}$ -phosphate transfections frequently causes neuronal cell death, which is clearly detectable by microscopic examination prior to immunostaining experiments (Troubleshooting 2). One should avoid conducting immunostaining experiments, capturing images, and performing quantitative analyses using poor quality of neurons because it might negatively impact precise interpretation of results. Importantly, a typical confocal microscopic imaging experiment should show that a number of synaptic vesicle marker proteins (e.g., VGLUT1, VGAT, synaptophysin) are distributed and concentrated into presynaptic boutons, which in turn are identified by their immunostaining for well-established marker proteins (e.g., Bassoon, Piccolo, RIM1), used to mark anatomically defined presynaptic active zones.

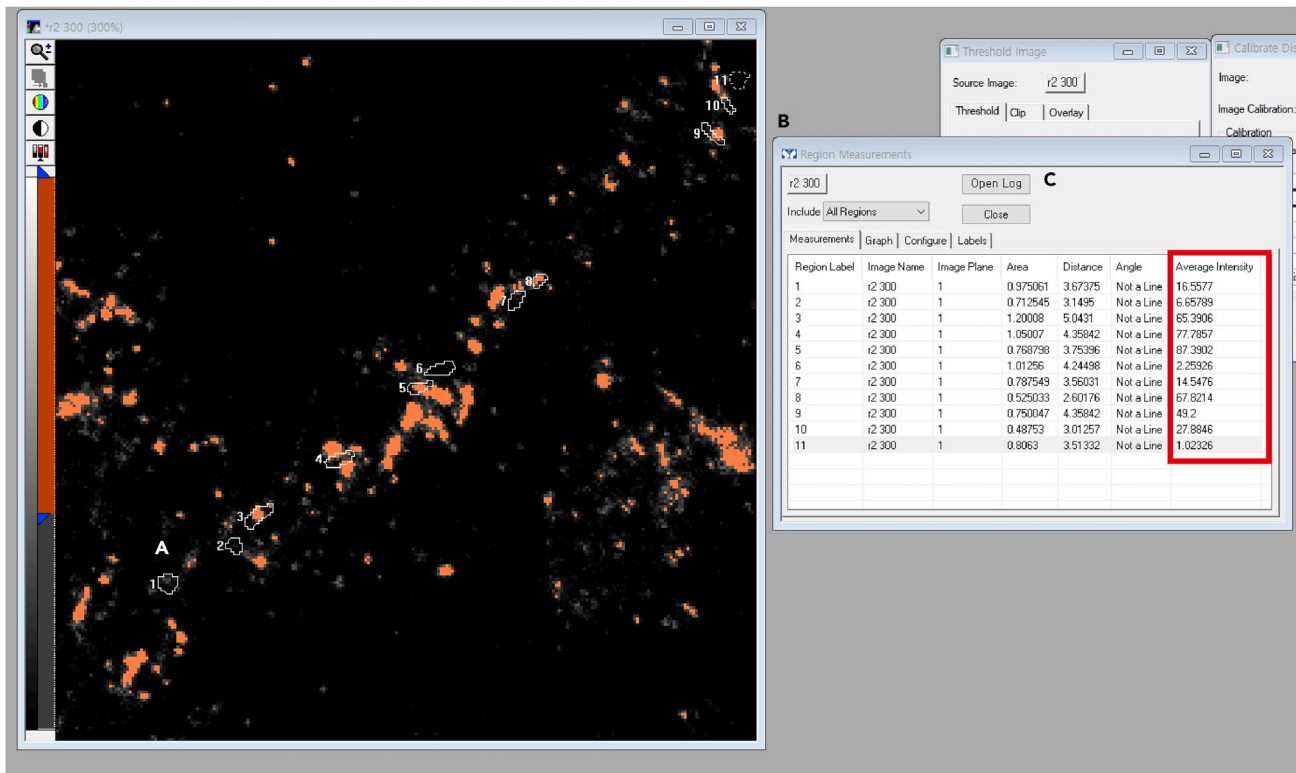
Although heterogeneity is unavoidable in cultured neuron experiments, it can be reduced by repeating multiple independent experiments under the same conditions. In addition, results should be quantified by researchers who are blinded to the experimental conditions and are not involved in preparing the cultured neurons, performing the infections or transfections, or archiving fluorescence images.

## QUANTIFICATION AND STATISTICAL ANALYSIS

### Measurement of Presynaptic Bouton Length

1. Average “Distance” values correspond to the length of a presynaptic bouton (see Table 1). This averaged value is obtained from a single image, which is considered an n-value of 1 in statistical evaluation. The mean value is the overall average of values averaged from individual experiments and should be based on at least 15 images from three or more independent experiments.





**Figure 11. Measuring the Percentage of VGLUT1-Venus and Bassoon Colocalization**

- (A) Loading the ROI.  
(B) Opening a Region Measurements window.  
(C) Exporting the files to the Microsoft excel.

primary cultured neurons have contributed to current models of synaptic assembly, particularly at presynaptic nerve terminals (Ahmari et al., 2000; Bamji et al., 2003; Ziv and Garner, 2004).

Although our immunostaining analyses using VGLUT1-mVenus and Bassoon (a marker for the presynaptic active zone) clearly enable us to dissect detailed molecular mechanisms responsible for clustering and confining synaptic vesicles in presynaptic bouton structures, it is impossible to discriminate “orphan” sites—in which postsynaptic apposition with the functional apparatus for neurotransmitter release is lacking—from *bona fide* synaptic sites (Krueger et al., 2003). Thus, implementing complementary functional imaging and/or electrophysiology approaches is critical. In addition, the magnitude of VGLUT1-mVenus fluorescence diffusion, quantified as the length of the major axis of each puncta, has been well established as a proxy for vesicle clustering. Thus, live-cell imaging analyses could be employed to precisely determine the dynamics of synaptic vesicle material influx and efflux in presynaptic structures. High-resolution electron microscopic analyses are sometimes warranted to further investigate possible anatomical defects that cannot be discerned by light microscopy, as we have done in our recent studies (Han et al., 2020a; Han et al., 2020b).

## TROUBLESHOOTING

### Problem 1

Poor neuron cell culture quality.

### Potential Solution

There are multiple points in the procedure that could contribute and need to be assessed.



**Table 1. An Example of Quantified Data, Exported to a Microsoft Excel Sheet**

Region Label	Distance
1	1.6715037
2	1.291841439
3	1.715789613
4	1.902360688
5	1.946210866
6	2.085730542
7	1.561298291
8	1.60278213
9	1.355586534
10	1.355586534
11	2.819662552
12	1.291841439
13	1.26247757
14	2.212251314
15	1.042865271
16	2.498827777
average	1.726038516

First, precisely timed pregnant female rats or mice should be used. Embryos staged at E17 (in the case of mice) or E18 (in the case of rats) are used in experiments, and 8–12 embryos are normally obtained from a single pregnant female rodent. Errors during examinations for a vaginal plug in the female, palpitation or visual confirmation of pregnancy could result in failure to obtain precisely timed pregnant females. The use of such early- or later-stage embryos could contribute to the poor quality of neuronal cultures. Although some laboratories have reported the use of P0 mouse pups, we find that cultured neurons obtained from embryos are higher quality.

Second, preparation of coverslips is a crucial step for obtaining high-quality cultured neurons. If nitric acid has been used for an extended period of time, it should be replaced with a new batch of concentrated nitric acid.

Third, poly-D-lysine solution prepared in borate buffers should be re-checked. We have had some success in adding laminin to promote the attachment and differentiation of plated neurons.

Lastly, neuron culture medium contains several components that should be carefully monitored because of lot-to-lot variability (e.g., fetal bovine serum, GlutaMAX, and B27). GlutaMAX should be aliquoted and thawed in a refrigerator for 12–16 h before use. Notably, the quality of the B27 supplement is crucial for obtaining pure and healthy neuronal populations with little or no glial contamination; thus, specific lots should be tested before using.

## Problem 2

Low transfection efficiency in cultured primary neurons.

### Potential Solution

An experience researcher immersed in the  $\text{Ca}^{2+}$ -phosphate transfection method will normally obtain a fair number of transfected neurons on a single coverslip with low cell toxicity. Usually, one should expect to obtain around 100–1,000 transfected neurons on a coverslip placed in a 12-well plate (at a density of  $3 \times 10^5$  neurons; transfection efficiency is around 0.033% to ~0.33%). However, this

method has been notorious for its low transfection efficiency and poor reproducibility, reflecting its sensitivity to pH, temperature and incubation time (Jiang and Chen, 2006). The key is to develop the pipetting skills necessary to successfully form homogeneous precipitates with  $\text{Ca}^{2+}$  ions in phosphate buffer that fall “snow-like” onto neurons during the incubation period.

The concentration, quality, and size of plasmid DNA are all capable of affecting transfection efficiency. Thus, it is often necessary to empirically optimize the transfection protocol prior to use, which may include increasing the incubation time during  $\text{Ca}^{2+}$  phosphate–DNA complex preparation. Other transfection methods, including lipofection, electroporation or nucleofection, could be employed as alternatives to  $\text{Ca}^{2+}$ -phosphate transfection method in cases where low transfection efficiency continues to be problematic.

**Note:** Although electroporation and nucleofection yield very high transfection efficiencies, they also require specialized equipment and expensive reagents. Moreover, these methods are not ideal for adherent cell cultures.

### RESOURCE AVAILABILITY

#### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jaewon Ko ([jaewonko@dgist.ac.kr](mailto:jaewonko@dgist.ac.kr)).

#### Materials Availability

This study did not generate new unique reagents.

#### Data and Code Availability

Not applicable.

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### AUTHOR CONTRIBUTIONS

J.W.U. and J.K. conceived and supervised the project. K.A.H. conducted the experiments. J.W.U., K.A.H, S.-Y.C., and J.K. analyzed data. J.W.U. and J.K. wrote the manuscript. All authors read and approved the final version of the manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

### REFERENCES

- Ahmari, S.E., Buchanan, J., and Smith, S.J. (2000). Assembly of presynaptic active zones from cytoplasmic transport packets. *Nat. Neurosci.* 3, 445–451.
- Bamji, S.X., Shimazu, K., Kimes, N., Huelsenken, J., Birchmeier, W., Lu, B., and Reichardt, L.F. (2003). Role of beta-catenin in synaptic vesicle localization and presynaptic assembly. *Neuron* 40, 719–731.
- Han, K.A., Lee, H.Y., Lim, D., Shin, J., Yoon, T.H., Lee, C., Rhee, J.S., Liu, X., Um, J.W., Choi, S.Y., et al. (2020a). PTPsigma controls presynaptic organization of neurotransmitter release machinery at excitatory synapses. *iScience* 23, 101203.
- Han, K.A., Lee, H.Y., Lim, D., Shin, J., Yoon, T.H., Liu, X., Um, J.W., Choi, S.Y., and Ko, J. (2020b). Receptor protein tyrosine phosphatase delta is not essential for synapse maintenance or transmission at hippocampal synapses. *Mol. Brain* 13, 94.
- Jiang, M., and Chen, G. (2006). High  $\text{Ca}^{2+}$ -phosphate transfection efficiency in low-density neuronal cultures. *Nat. Protoc.* 1, 695–700.
- Krueger, S.R., Kolar, A., and Fitzsimonds, R.M. (2003). The presynaptic release apparatus is functional in the absence of dendritic contact and highly mobile within isolated axons. *Neuron* 40, 945–957.
- Ziv, N.E., and Garner, C.C. (2004). Cellular and molecular mechanisms of presynaptic assembly. *Nat. Rev. Neurosci.* 5, 385–399.