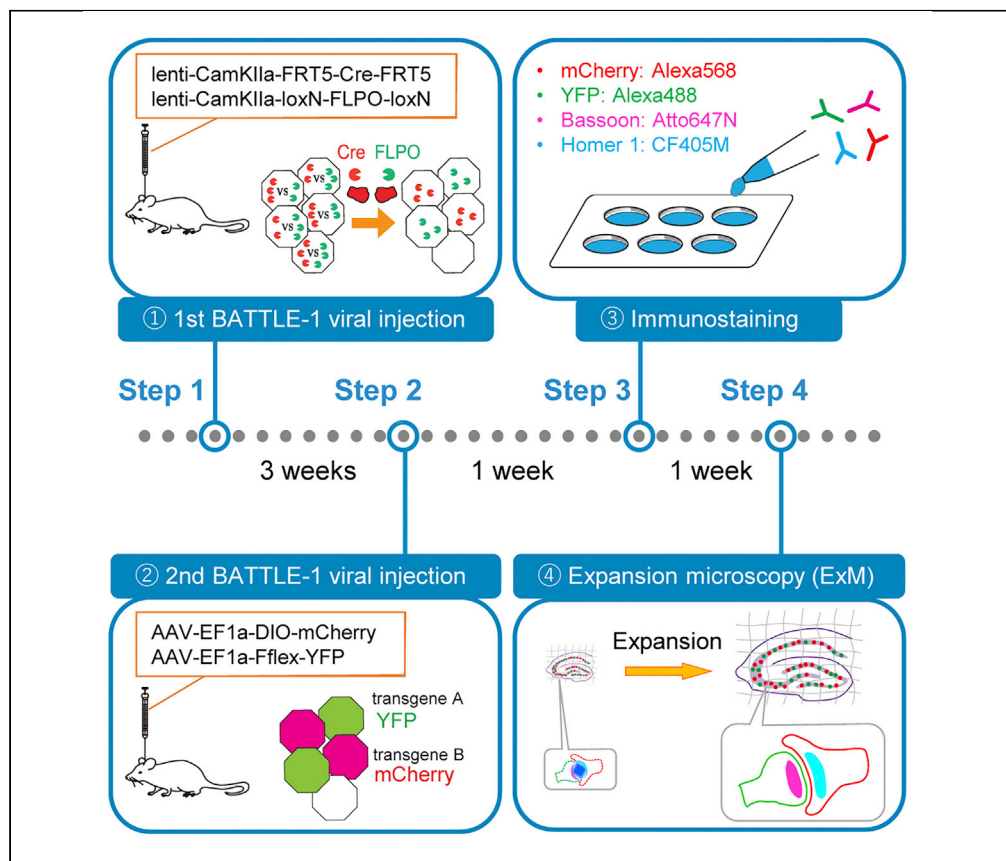## Protocol

Protocol for BATTLE-1EX: A High-Resolution Imaging Method to Visualize Whole Synaptic Structures and their Components in the Nervous System

Akitoshi Inoue,
Takuya Kobayashi,
Hirokazu Hirai,
Noriko Kanaya,
Keigo Kohara

koharake@hirakata.kmu.ac.jp

HIGHLIGHTS

BATTLE-1EX enables 3D high-resolution imaging of whole synapses in the hippocampus.

Split-tunable allocation of transgenes by competition between two recombinases

Entire synaptic morphologies can be expanded without changing protein placement

Localizations of synaptic proteins can be visualized in whole synaptic structures

This protocol describes BATTLE-1EX, which is a combined method of BATTLE-1 and expansion microscopy to obtain high-resolution imaging of whole synaptic structures and their components of hippocampal neural circuits. BATTLE-1 uses two genetically engineered recombinase proteins and competition between two recombinases that can be independently titrated, resulting in a tunable proportion of mCherry+/YFP− and YFP+/mCherry− cells. As a combinational method, BATTLE-1EX has the potential to visualize and dissect whole synaptic structures in numerous regions in the brain.

STAR Protocols

Protocol

Protocol for BATTLE-1EX: A High-Resolution Imaging Method to Visualize Whole Synaptic Structures and their Components in the Nervous System

Akitoshi Inoue,[1] Takuya Kobayashi,[1] Hirokazu Hirai,[2,3] Noriko Kanaya,[4] and Keigo Kohara[4,5,6,*]

[1]Department of Medical Chemistry, Kansai Medical University, Graduate School of Medicine, Hirakata, Osaka 573-1010, Japan

[2]Department of Neurophysiology and Neural Repair, Gunma University Graduate School of Medicine, Maebashi, Gunma 371-8511, Japan

[3]Research Program for Neural Signaling, Division of Endocrinology, Metabolism and Signal Research, Gunma University Initiative for Advanced Research, Maebashi, Gunma 371-8512, Japan

[4]Department of Cellular and Functional Biology, Institute of Biomedical Science, Kansai Medical University, Hirakata, Osaka 573-1010, Japan

[5]Technical Contact

[6]Lead Contact

*Correspondence: koharake@hirakata.kmu.ac.jp
https://doi.org/10.1016/j.xpro.2020.100166

SUMMARY

This protocol describes BATTLE-1EX, which is a combined method of BATTLE-1 and expansion microscopy to obtain high-resolution imaging of whole synaptic structures and their components of hippocampal neural circuits. BATTLE-1 uses two genetically engineered recombinase proteins and competition between two recombinases that can be independently titrated, resulting in a tunable proportion of mCherry+/YFP− and YFP+/mCherry− cells. As a combinational method, BATTLE-1EX has the potential to visualize and dissect whole synaptic structures in numerous regions in the brain.
For complete details on the use and execution of this protocol, please refer to Kohara et al. (2020).

BEFORE YOU BEGIN

Dissection of the synaptic structures and their components is crucial to understanding the basal and functional mechanisms of the brain. To visualize the whole structure of synaptic connections, it is quite difficult, but presynaptic and postsynaptic neurons are required to be labeled sparsely in the highly dense neuronal circuit by different colors. To date, considerable efforts have been made to visualize and dissect whole synaptic structures using light microscopy, but most of these studies only succeeded in labeling half of the synaptic structures (Nimchinsky et al., 2002; Chen et al., 2015; Luo et al., 2016) such as spines or presynaptic terminals alone. BATTLE-1 technology (Kohara et al., 2020) enables split-tunable allocation of transgenes by recombinase "battle." In this protocol, Cre and FLPO are expressed under the control of CamKIIa promoter which drives expression in the excitatory neurons (Benson et al., 1992) by lentivirus injection into the mouse hippocampus (Figure 1A). The Cre transgene flanked by two FRT5 is designed to be a target of the FLPO recombinase (Schlake and Bode, 1994). The FLPO transgene flanked by two loxN is designed to be a target of the Cre recombinase (Livet et al., 2007). In this configuration, Cre is oriented to excise the FLPO transgene, which is then oriented to excise the Cre transgene. Three weeks later, Cre-dependent AAV expressing mCherry and FLPO-dependent one expressing YFP are injected into the same regions. Cre and FLPO "battle" result in splitting allocation of YFP and mCherry
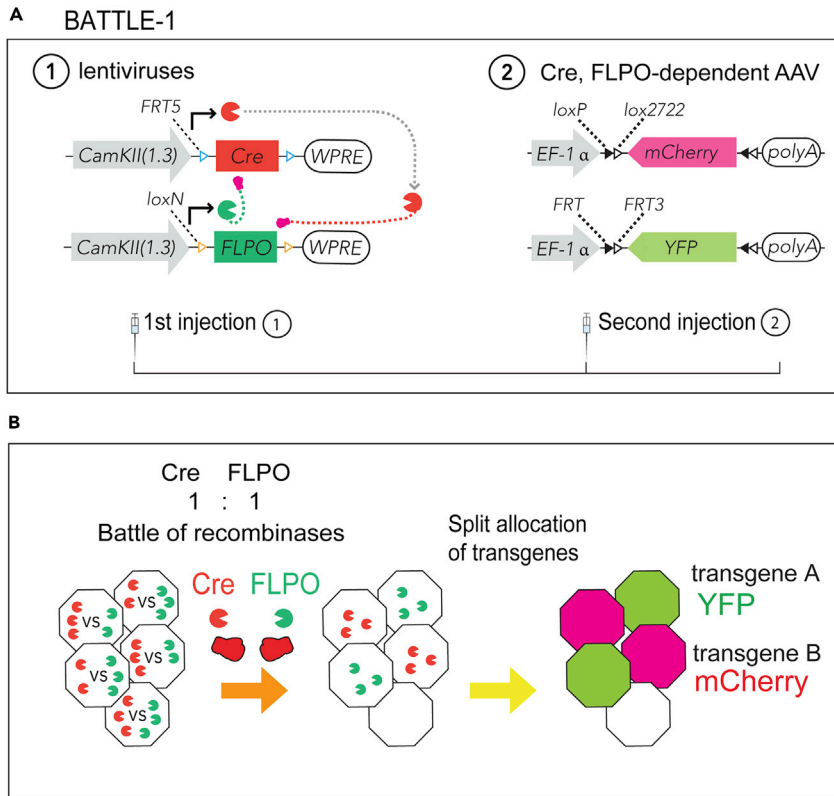
Figure 1. BATTLE-1: Split-Tunable Allocation of Transgenes by Recombinase "Fights"

(A) In the BATTLE-1 technology, Cre and FLPO are expressed under the control of CamKIIa promoter. The Cre transgene flanked by two FRT5 is designed to be a target of the FLPO recombinase. The FLPO transgene flanked by two loxN is designed to be a target of the Cre recombinase. Cre- and FLPO-dependent AAVs expressing mCherry and YFP, respectively, were used.

(B) Illustration shows the battle of recombinases and the BATTLE-1 system. After the first injection of mixed viruses, Cre and FLPO started recombinase battles in each infected neuron. Then, only the winning recombinase survived to induce its specific mutually exclusive allocation of transgenes (mCherry or YFP). Adapted from Figures 1A and 1H in Kohara et al. (2020).

(Figure 1B). YFP and mCherry expression is easily tunable by changing the ratio of the Cre and FLPO expression.

> *Note:* Expansion microscopy is a technique developed in the Ed Boyden lab that physically enlarged samples through an isotropic chemical expansion process (Chen et al., 2015; Tillberg et al., 2016; Asano et al., 2018). In this method, immunostained proteins are anchored to the swellable gel by cross-linking molecule (acryloyl-X). Proteinase K digestion and gel swelling by water result in sample expansion linearly by about four times without changing protein placement. In our case, a lens with a diffraction limit of 280 nm (60×, 1.2 numerical aperture water objective from Olympus) would obtain an effective resolution of 280 nm/4 = 70 nm.

> *Note:* Using BATTLE-1EX, whole synaptic structures consisting of YFP-positive presynaptic terminals from the dentate gyrus and mCherry-positive postsynaptic spines of CA3 pyramidal neurons can be clearly visualized. Furthermore, the localizations of Bassoon and Homer, which are presynaptic and postsynaptic proteins, respectively, can also be simultaneously identified in whole synaptic structures.

Preparation of Lentiviruses and AAVs for BATTLE-1

⏱ Timing: several weeks

Note: Perform Lentivirus Preparation of BATTLE-1 according to Torashima et al., 2006.

1. Increase plasmids of lentivirus BATTLE-1 (lentivirus-CamKIIa (1.3 kb)-FRT5-iCre-FRT5-WPRE and lentivirus-CamKIIa (1.3 kb)-loxN-FLPO-loxN-WPRE) by stbl3 competent cells using an endotoxin-free Giga prep kit or endotoxin-free Mega prep kit (20–200 μg).
2. Virus packaging: Seed HEK293T cells (cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 atmosphere) 2–4 × 10^6 cells in a 10-cm culture dish 24 h before transfection.
3. Dilute vectors: Mix lentiviral packaging vectors (Invitrogen, 10 μg), and single lentiviral vector created above (10 μg) in sterile water to a total volume of 450 μL; then add 50 μL of 2.5 M $CaCl_2$ and vortex. Drop 500 μL of 2× HEPES-buffer while vortexing and immediately add precipitate to the cultures before incubating overnight (14 h–18 h).
4. Wash cultures twice with PBS and incubate additionally for 24 h.
5. Filtrate collected medium through 0.22-μm membrane and centrifuge at 120,000 × *g* for 1.5 h at 4°C using a swinging bucket rotor.
6. Suspend precipitated virus in 45 μL of PBS and store at −80°C until use.
7. Determine each titer of lentiviruses of BATTLE-1 using Lenti-X™ qRT-PCR Titration Kit (Takara).

Note: AAV (serotype 9) preparation of BATTLE-1 can be done by availing of the AAV service offered by Addgene.

8. Increase AAV backbone vectors (AAV-EF1a-Fflex-EYFP and AAV-EF1a-DIO-mCherry) by stbl3 competent cells using an endotoxin-free Giga prep kit or endotoxin-free Mega prep kit (20–200 μg).
9. Avail of the AAV service offered by Addgene.

Note: Lentiviral and AAV backbone vectors are listed in the Key Resources Table. See Resource Availability section for requirement of vectors constructed in Kohara et al. (2020).

⏸⏸ **Pause Point:** Lentiviruses and AAVs of BATTLE-1 (5 μL each) were aliquoted on ice in the safety cabinet and stored in a freezer box at −80°C.

⚠ CRITICAL: Using stable competent cells like stbl3 (Invitrogen) is essential to prevent unexpected recombination of lentiviral plasmid vector. We also recommend growing bacteria on the agar plate at 32°C, small-scale growth (5 mL) at 32°C, and large-scale growth (about 2 L) at 37°C for the same purpose.

Note: We confirm the existence of LTRs in lentiviral plasmid vector using restriction digestion (Smal).

Preparation of Sharp Glass Pipettes

⏱ Timing: 1 h

10. Make sharp glass pipettes by using micropipette puller and sharp glass pipettes (Narishige GD-1: 1-mm diameter glass pipette filled with filament).
11. Break the tip of a glass pipette by pushing it slowly to clean the surface of box (for example, top lid of pipette man tip rack).

Table 1. Percentage of mCherry-, YFP-, and Double-Positive Cells Induced by BATTLE-1 with Different Ratios of Cre and FLPO

	mCherry-Positive Cells (%) (Mean ± SEM)	YFP-Positive Cells (%) (Mean ± SEM)	mCherry, YFP− Double-Positive Cells (%) (Mean ± SEM)	Viral Titers of BATTLE-1 Lentiviruses	Total Cell and Mouse Numbers
Cre:FLPO 1:1	61.3 ± 5.8	38 ± 5.8	0.7 ± 0.2	5×10^8 copies each	n = 827, N = 5
Cre:FLPO 10:1	92 ± 1.4	7.4 ± 1.6	0.6 ± 0.4	5×10^8, 5×10^7 copies each	n = 876 N = 3
Cre:FLPO 100:1	98.7 ± 0.2	1.1 ± 0.2	0.2 ± 0.1	5×10^8, 5×10^6 copies each	N = 1,153, N = 3

The BATTLE-1 method mostly induced split-tunable expression of mCherry and YFP in dentate granule neurons in the hippocampus. The data of Cre:FLPO = 1:1, and Cre:FLPO= 1:0.01 were adopted from Figures 1D and 1G in Kohara et al., 2020.

12. Confirm that the diameter of the tip of the sharp glass pipette is between 30 and 40 μm through the binocular microscope (Narishige).
13. Keep dozens of modified sharp glass pipettes in a home-made box with sticky rubber.

Setting Coordination of the Stereotaxic Injection Site

⏱ Timing: 30 min

14. Determine stereotaxic injection site of the experiment using a ruler and pencil, according to the book "The Mouse Brain in Stereotaxic Coordinates" (Flanklin and Paxinos, 2008, coronal sections).
15. Record anterior-posterior (AP) position, medio-lateral (ML) position, and dorso-ventral (DV) position of injection site of the experiment.

Preparation of BATTLE-1 Lentivirus Injection Solution

⏱ Timing: 15 min

16. Prepare same titer viral solution of lentivirus-CamKIIa (1.3 kb)-FRT5-iCre-FRT5-WPRE and lentivirus-CamKIIa (1.3 kb)-loxN-FLPO-loxN-WPRE on ice just before the first injection experiment.
17. Mix same volume of their lentiviruses on ice (lentivirus-CamKIIa (1.3 kb)-FRT5-iCre-FRT5-WPRE: lentivirus-CamKIIa (1.3 kb)-loxN-FLPO-loxN-WPRE = 1:1). Keep it on ice in the small cooler box.

Note: We prepare 1 μL mixed lentiviruses (lenti-CamKIIa-FRT5-Cre-FRT5:lenti-CamKIIa-loxN-FLPO-loxN = 1:1 [each virus consisted of 5×10^8 copies]) for single injection. Lower titer of lentiviruses also works. In the first injection experiment, do dosage effect experiment using the same titer of 1× lentivirus-mix solution (Cre:FLPO = 1:1) and 10× diluted lentivirus-mix solution (Cre:FLPO = 0.1:0.1). Determine the better concentration of lentiviruses in your experiment by checking the result of dosage effect experiment. You can save the cost of the experiments using the lower dosages of lentiviruses.

Alternatives: If you prefer different splitting situations in which postsynaptic neurons are sparsely labeled with YFP, and presynaptic terminals are densely labeled with mCherry (Table 1), you can use a lentivirus-mix solution with different ratios (Cre:FLPO=10:1, or 100:1).

Preparation of BATTLE-1 AAV Injection Solution

⏱ Timing: 15 min

18. Prepare mixed AAV solution (AAV-EF1a-DIO-mCherry:AAV-EF1a-DIO-YFP = 1:1, titer: 1.43×10^{13} copies/mL each) on ice just before the second injection experiment. Keep it on ice in the small cooler box.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
chicken polyclonal antibody to GFP	Abcam	ab13970
rat monoclonal antibody to RFP	Chromotek	5f8-100
mouse monoclonal antibody to Bassoon	Abcam	ab82958
rabbit polyclonal antibody to Homer1	SYSY	#160003
anti-chicken donkey antibody conjugated with Alexa488 dye	Jackson	#703-545-155
anti-rat donkey antibody conjugated with Alexa568 dye	Abcam	#ab175710
anti-mouse goat antibody conjugated with Atto647N dye	Rockland	#50185
anti-rabbit goat antibody conjugated with CF405M	Biotium	#20373
Bacterial and Virus Strains		
lentivirus-CamKIIa (1.3 kb)-FRT5-iCre-FRT5-WPRE	Kohara et al. (2020)	N/A
lentivirus-CamKIIa(1.3 kb)-loxN-FLPO-loxN-WPRE	Kohara et al. (2020)	N/A
AAV-EF1a-DIO-mCherry plasmid vector	Addgene	#50462
AAV-EF1a-Fflex-EYFP	Kohara et al. (2020)	N/A
Lentiviral packaging vectors (pLP1, pLP2 (pRev), pLP/VSVG)	Invitrogen	K4975-00
One Shot™ Stbl3™ Chemically Competent *E. coli*	Invitrogen	C737303
Experimental Models: Cell Lines		
HEK293T cells	ATCC	CRL-11268
Experimental Models: Organisms/Strains		
Mouse: C57BL6J	CLEA Japan	n/a
Chemicals, Peptides, and Recombinant Proteins		
SmaI	New England Biolabs	R0141S
Dulbecco's modified Eagle's medium	Sigma	D6546
Penicillin-streptomycin-glutamine (100×)	Invitrogen	#10378-016
FBS	Invitrogen	#26140079
Sodium Acrylate	Sigma	#408220
Acrylamide	Sigma	A9099
N,N′-Methylenebisacrylamide	Sigma	M7279
4-Hydroxy-TEMPO	Sigma	#176141
Ammonium Persulfate (APS)	Sigma	A3678
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma	T7024
Acryloyl-X SE	Invitrogen™	A20770

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Proteinase K	New England Biolabs	P8107S
Triton X-100 Polyethylene Glycol Mono-4-octylphenyl Ether	Nacalai	#12967-45
Tween-20	Santa Cruz Biotechnology	#23926-35
Tris (hydroxymethyl) aminomethane	Nacalai	#35401-25
EDTA (2NA)	FUJIFILM Wako	#345-01865
Paraformaldehyde (PFA), powder	Nacalai	#26126-25
Phosphate Buffered Saline (PBS) Tablets, pH 7.4	TaKaRa	T9181
HEPES	FUJIFILM Wako	#342-01375
Guanidine Hydrochloride	FUJIFILM Wako	#072-05001
Trisodium Citrate	FUJIFILM Wako	#203-13605
Sodium azide	Nacalai	#31208-82
Mineral Oil	Nacalai	#23306-84
Tarivid ophthalmic ointment 0.3%	Santen	N/A
Critical Commercial Assays		
EndoFree Plasmid Mega Kit	QIAGEN	#12381
Lenti-X™ qRT-PCR Titration Kit	Takara	#631235
Software and Algorithms		
FV31S-SW	OLYMPUS	N/A
Imaris 9.1	Bitplane	N/A
Other		
Steriflip-GP filter (0.22-μm) unit	Millipore	SCGP00525
FV3000 Confocal Laser Scanning Microscope	OLYMPUS	N/A
S9i stereo microscope	Leica	N/A
10-cm cell culture dishes	Falcon	#353003
Passive gas anesthesia mask	WPI	#505147
MEDI SHEET	SANSYO	#23-1079
Desk stand Magnifire (×5)	SANWA DIRECT	#400-CAM019
Micro Drill	WPI	#503598
Mineral Oil	Nacalai	#23306-84
Isodine 10%	mMeiji	2612701Q3318
Isoflurane	FUJIFILM	099-06571
Midazolam	SANDOZ	N/A
DOMITOR	ZENOAQ	N/A
Vetorphale	Meiji	v024094
Foam Cleaning Heiter-1000 (Commercial Chlorine Disinfecting Cleaner)	Kao	N/A
MicroFil 28 g	WPI	MF28G67-5
HAMILTON syringe	HAMILTON	#1701RN
DUAL FERRULE, RN ADAPTOR, 1MM	HAMILTON	#55750-01
Glass Capillary with Filament	NARISHIGE	GD-1
Micropipette Puller	SUTTER INSTRUMENT	P-97/IVF
Microforge	NARISHIGE	MF-900

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Stereotaxic system	WPI	TAXIC600
SYS-MICRO4	WPI	SYS-MICRO4
Small scissors	Hammacher	HSB 002-09
Tweezers	Hammacher	HSC 184-10
Small surgical clamp	SANSYO	#91-3101
Hemostatic forceps	SANSYO	F-2A
Threaded suture needle	Natsume Seisakusyo	CE1440N2NT
NARCOBIT-E (anesthetic machine)	Natsume Seisakusyo	KN-1071-1
Vapor Guard	VetEquip	#931401
Cryostat	Leica	CM3050 S
Peristaltic pump	ATTO	SJ-1211 2-H
Microtome replacement razor	FEATHER Safety Razor	C35
Silicone Rubber Sheet (Thickness 0.5 mm)	TOGAWA RUBBER	K-125
Super Frost Slide Glass Pink	MATSUNAMI	#2-150-03
NEO Cover glass 25 × 60	MATSUNAMI	#83-0225-3
NEO Cover glass 24 × 60	MATSUNAMI	#83-0217-3
NEO Cover glass 24 × 32	MATSUNAMI	#83-0211-3
OCT Compound	Sakura Finetek Japan	#45833

MATERIALS AND EQUIPMENT

2× HEPES-Buffer	Final Concentration	Amount
HEPES	50 mM	1.2 g
$Na_2HPO_4 \cdot 2H_2O$	350 mM	0.027 g
NaCl (5 M)	280 mM	5.6 mL
NaOH	n/a	Adjust pH to 7.05
ddH_2O	n/a	Adjust to 100 mL
Total	n/a	100 mL

Sterilize by filtration through a 0.22-μm filter. Store at −20°C.

Mixed Anesthetic	Final Concentration	Amount
Domitor (medetomidine 1 mg/mL)	0.06 mg/mL	0.3 mL
Dormicum (midazolam 5 mg/mL)	0.8 mg/mL	0.8 mL
Vetorphale (butorphanol 5 mg/mL)	1 mg/mL	1 mL
Saline	n/a	2.9 mL
Total	n/a	5 mL

Prepare right before use.

Citrate Buffer pH6.0	Final Concentration	Amount
trisodium citrate	10 mM	294 mg
Tween 20 (10%)	0.05%	0.5 mL
ddH_2O	n/a	up to 100 mL
Total	n/a	100 mL

Store at 25°C for 3 months or 4°C for longer storage.

Blocking Buffer	Final Concentration	Amount
BSA	2.50%	2.5 g
Triton X-100 (10%)	0.10%	1 mL
ddH$_2$O	n/a	99 mL
Total	n/a	100 mL

Store at 4°C for 1 month.

High Salt PBS	Final Concentration (mM or μM)	Amount
10× PBS	n/a	10 mL
NaCl (5 M)	0.4 M	8 mL
ddH$_2$O	n/a	82 mL
Total	n/a	100 mL

Store at 25°C.

AcX Buffer	Final Concentration	Amount
Acryloyl-X SE (10 mg/mL, DMSO)	0.1 mg/mL	10 μL
1× PBS	n/a	990 μL
Total	n/a	1 mL

Store 10 mg/mL Acryloyl-X SE as aliquots of 20 μL each at −20°C for at least 1 month. Add PBS right before use.

Monomer Solution	Final Concentration (After Making Gelling Solution)	Amount
Sodium acrylate (4M)	900 mM	2.25 mL
Acrylamide (7M)	350 mM	0.5 mL
N,N'-Methylenebisacrylamide (130 mM)	9.75 mM	0.75 mL
NaCl (5 M)	2 M	4 mL
10× PBS	n/a	1 mL
ddH$_2$O	n/a	0.9 mL
Total	n/a	9.4 mL

Store as aliquots of 1 mL each at −20°C for at least 1 month.

Gelling Solution	Final Concentration	Amount
Monomer solution	n/a	188 μL
4-hydroxy-TEMPO (0.5%)	0.01%	4 μL
TEMED (10%)	0.2%	4 μL
APS (10%)	0.2%	4 μL
Total	n/a	200 μL

Prepare at 4°C right before the gelation step.

Digestion Buffer	Final Concentration	Amount
Tris-HCl pH8 (1 M)	50 mM	0.5 mL
EDTA (0.5 M)	1 mM	0.02 mL

(Continued on next page)

Continued

Digestion Buffer	Final Concentration	Amount
Guanidine Hydrochloride (8 M)	0.8 M	1 mL
Triton X-100 (10%)	0.5%	0.5 mL
ddH$_2$O	n/a	7.88 mL
Proteinase K (800 units/mL)	8 units/mL	0.1 mL
Total	n/a	10 mL

Store digestion buffer (without Proteinase K) as aliquots at −20°C for a few months. Add Proteinase K right before use.

STEP-BY-STEP METHOD DETAILS

> ⏲ Timing: 1 month in total

A timeline of all main steps is summarized in the Graphical abstract. Briefly, it takes several hours for lentiviral and AAV injections. Three-week intervals between the first lentiviral and second AAV injections, and a one-week interval between the second AAV injection and brain sampling are needed. It takes 1 week for immunostaining and ExM.

First Stereotaxic Injection of BATTLE-1 Lentivirus into Mouse Brain

> ⏲ Timing: 2–4 h

Perform stereotaxic injection of viruses according to Kohara et al. (2014) and Kohara et al. (2020).

⚠ CRITICAL: Perform stereotaxic injection of BATTLE-1 lentivirus in the Biohazard room P2.

1. Prepare surgery set up in the safety cabinet in P2 biosafety room.
   a. Place the stereotaxic injection systems on the safety cabinet.
   b. Prepare the surgery tools, suture needle with thread, and microdrill on the safety cabinet.
   c. Prepare skin marker, cotton swabs, and ointment pot (a box for used pipette tip attached with viral liquid) added with commercial chlorine disinfecting cleaner (Hypochlorous acid >0.1%).
   d. Prepare pipette man (P2, P20, P200) and filtered tips on the safety cabinet.
   e. Put 50 mL autoclaved PBS and isodine.
   f. Place the anesthetic machine for isoflurane beside the safety cabinet and fill with isoflurane.
   g. Connect the tube between anesthetic machine and gasmask attached in stereotaxic injection system.
   h. Connect the tube between the extra isoflurane retrieve bottle and the gas mask attached in stereotaxic injection system.
   i. Place a small desk stand with a magnifying glass on the safety cabinet.
   j. Prepare small square parafilms (about 1 cm × 1 cm) for back-filling sharp glass pipette with virus solution.
   k. Place a small shaver on the side desk for cutting the skull hair of the mouse.
2. Prepare Hamilton syringe and sharp glass pipettes on the desk in P2 biosafety room.
   a. Place a 50-mL centrifuge tube filled with mineral oil in the tube stand on the desk.
   b. Prepare Hamilton syringe, sharp glass pipette box, and backfill syringe with sharp tube.
   c. Remove metal joint fitting of Hamilton syringe and place it on the desk.
   d. Fill metal joint fitting with mineral oil on the desk.
   e. Fill mineral oil on the upper lid of 50-mL centrifuge tube on the desk.
   f. Insert the tip of Hamilton syringe without metal joint fitting (but with plastic compression fitting) into the upper lid of 50-mL centrifuge tube filled with mineral oil.
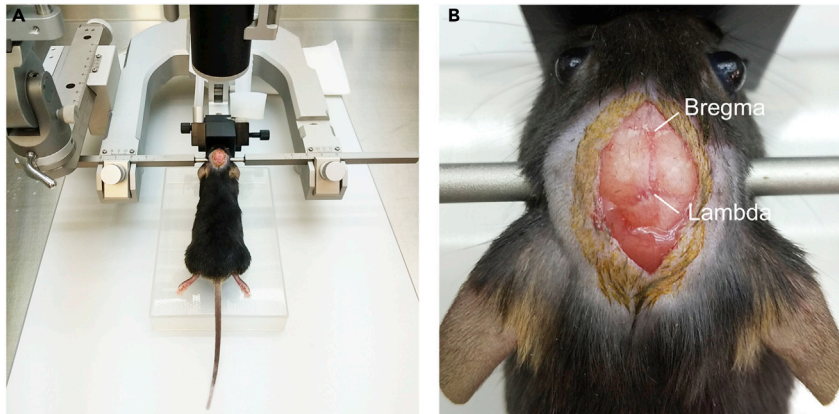
Figure 2. Stereotaxic Injection System and Anesthetized Head-Fixed Mouse
(A) A representative picture of the stereotaxic injection system and a head-fixed mouse. The mouse is anesthetized by 3% isoflurane and an incision made on the skull skin.
(B) The magnified image of (A). Bregma point and Lambda point are shown.

    g. Fill mineral oil in the Hamilton syringe and remove air in the Hamilton syringe by repeating plunger pushing and withdrawing.
    h. After removing the bubble air in the Hamilton syringe, connect the Hamilton syringe with metal joint fitting (avoid trapping any air).
    i. Backfill sharp glass pipette with mineral oil by using backfill syringe with sharp tube (avoid trapping any air bubbles in the sharp glass pipette).
    j. Connect carefully sharp glass pipette (filled with mineral oil) with Hamilton syringe (filled with mineral oil).
    k. Attach Hamilton syringe connected with sharp glass pipette to stereotaxic injector in the safety cabinet.
    l. Move attached Hamilton syringe to the highest position just above mouse head. (This position is probably the safest position during surgery to avoid your hand from unexpectedly touching a sharp glass pipette with virus.)
3. Anesthetize mouse (older than 8 weeks) in the box filled with 3% isoflurane.
4. Shave skull hair of anesthetized mouse using slim hair cutter.
5. Fixation of mouse head with ear bars in stereotaxic injection system (Figure 2A).
    a. Fix left ear bar in the appropriate position and unfixed right ear bar in stereotaxic injection system.
    b. Insert fixed left ear bar into left ear canal of mouse.
    c. Adjust mouse head direction in parallel to ground in that state.
    d. Insert right ear bar (in flexible state) around the right ear canal of mouse.
    e. When right ear bar is adequately inserted into right ear canal of mouse, mouse skull moves with movement of right ear bar.
    f. Fix the right ear bar when it is adequately inserted into the ear canal.
    g. Confirm that the mouse head can rotate vertically to the fixed ear bar.
    h. Fit gas anesthesia mask to mouse nose.
    i. Adjust gas anesthesia mask z-position at roughly good position.
6. Apply eye ointment by cotton swabs on mouse eyes to prevent dry eyes.
7. Sanitize mouse head hair with povidone iodine and cotton swabs.
8. Cover mouse body with a trimmed surgical paper.
9. Make an incision on the mouse head skin (Figure 2B).
10. Use small surgical clamps to expose skull bone and wipe jelly on the skull by cotton swab.
11. Mark points at Bregma and Lambda using skin marker.

12. Move and lower sharp glass pipette to just above the Lambda point by using magnifying stand glass (×5).
13. Contact the tip of sharp glass pipette with the surface of skull at Lambda point. Monitor the tip through magnifying stand glass (×5).
14. Record dorso-ventral (DV) position of Lambda point by reading scale of stereotaxic injection system.
15. Upper and move sharp glass pipette to Bregma.
16. Contact the tip of sharp glass pipette with the surface of skull at Bregma point. Monitor the tip through magnifying stand glass (×5).
17. Record DV position of Bregma point by reading scale of stereotaxic injection system.
18. When DV position of Bregma does not equal to that of Lambda, adjust the position of gas anesthesia mask.
19. Confirm that DV position of Bregma equals that of Lambda and record the anterior-posterior (AP) position and medio-lateral (ML) position of Bregma.
20. Move the sharp glass pipette to the injection site determined in the preparation step.
21. Mark the injection site using skin marker.
22. Drill slowly at marked injection site using microdrill. When the skull bone becomes thin, make a hole carefully using the bent tip of the needle.
23. Dig up the small bones at the injection site by the bent tip of the needle.
24. Remove blood by cotton swabs and apply sterilized PBS.
25. Place a small piece of parafilm on the ear bar.
26. Apply 2 μL of mixed lentiviruses on a small piece of parafilm.
27. Lower sharp glass pipette into the water drop of mixed lentiviruses.
28. Suck up 1.2–1.5 μL of mixed lentiviruses into a sharp glass pipette (speed: 90 nL/s) by pressing the button of microsyringe controller micro4 (as lentivirus is relatively unstable at room temperature (22°C–26°C), load only necessary volume of lentivirus solution for single injection).
29. Move the sharp glass pipette to the injection site and lower it to a position 0.3 mm deeper than the injection site and then return to injection site (make some space around the injection site for injection).
30. Inject 1 μL of mixed lentiviruses solution (speed: 30 nL/s).
31. Wait for 5 min.

   Note: Quick retrieving of a sharp glass pipette from the injection site just after injection probably causes spread of lentiviruses to the needle track.

32. Upper the sharp glass pipette.
33. Stitch skin of mouse head.
34. Place the mouse in a new cage and heat the cage by the heater sheet until the mouse becomes awake.
35. Wait for 3 weeks until the second AAV injection.

Second Stereotaxic Injection of BATTLE-1 AAV into Mouse Brain

   ⏱ Timing: 2–4 h

36. Prepare surgery set up in the safety cabinet in P2 biosafety room in the same manner as the first injection three weeks after the first surgery.
37. All processes are the same as the first injection except the following steps.
38. The skull bone at the injection site is usually thin because of the first injection. Make the injection hole by a vent needle.
39. Inject 1 μL of mixed AAV solution into the same injection site as the first one (speed: 30 nL/s)
40. Wait for 5 min.
41. Inject 1 μL of mixed AAV solution into the same injection again (speed: 30 nL/s)

42. The subsequent surgery processes are same as the first injection.
43. Wait for 1 week until the brain section.

⚠ CRITICAL: Lentivirus can be inactivated by 70% ethanol, but AAV is not inactivated by 70% ethanol. Use hypochlorous acid (>0.1%) to sterilize the used tip for AAV.

Preparation of Brain Sections

⏱ Timing: 3 days

44. Sampling: Anesthetize mice with an intraperitoneal administration (5 mL/kg body weight) of a mixed anesthetic (medetomidine, midazolam, and butorphanol), then perfuse transcardially with ice-cold 4% PFA in PBS using a peristaltic pump. Postfix removed brain in the same solution for 24 h, transfer to 30% sucrose in PBS, and embed in optimal cutting temperature compound. Freeze brain with isopentane and liquid nitrogen.

⏸⏸ Pause Point: Sample can be stored at −80°C until use.

45. Cryosection: Section brain sagittally 60-μm thick using cryostat (Leica). Transfer sections in PBS to the 12-well plate.

Note: The protocols for brain sections can be modified by target proteins and samples. We cut the brain 60 μm thick in this protocol, but thinner slices may be better for antibody penetration. Using a vibratome to cut the brain without freezing is also an alternative.

Note: Roughly check the fluorescence of the sections through the 12-well plate using inverted microscope to select sections for Expansion microscopy (ExM).

⏸⏸ Pause Point: Sample can be stored in PBS with 0.02% sodium azide at 4°C in the dark until use.

Immunostaining

⏱ Timing: 1 week

The protocols for immunostaining can be modified by your target proteins and samples. For immunostaining of the free-floating sections, we use parafilm well plate that was made by pressing a magnetic stirrer bar on parafilm-covered empty chip tray (Figure 3A and 3B). We drop 100 μL buffer for one brain section in the well and use a paintbrush to transfer sections between the wells (Figure 3C). To avoid drying samples during incubation, add water to the bottom of the chip tray.

46. Antigen retrieval: incubate freely floating sections in citrate buffer at 80°C for 30 min in a 1.5-mL tube, and then allowed to cool at room temperature (22°C–26°C) slowly. The sections were washed five times with PBS for 5 min.

Note: Rough trimming of the sections by Iris scissors before immunostaining is recommended to save reagent.

Note: The antigen retrieval step is optional but showed strong effect on Bassoon immunostaining in our experiment.

47. Blocking: Incubate sections in blocking buffer in the parafilm well plate for 1 h at room temperature (22°C–26°C) with shaking.
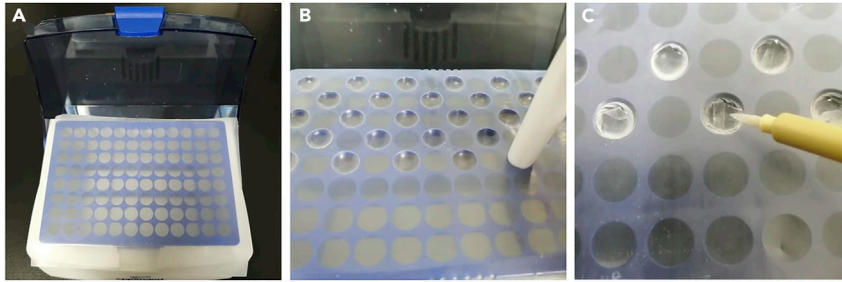
Figure 3. Parafilm Well Plate for Immunostaining
(A) Cover parafilm on the empty chip tray.
(B) Press a magnetic stirrer on parafilm to make wells.
(C) Use a paintbrush for sample transfer between wells.

48. Primary antibody staining: Incubate sections with primary chicken polyclonal antibody to GFP (1:500), rat monoclonal antibody to RFP (1:100), mouse monoclonal antibody to Bassoon (1:100), and rabbit polyclonal antibody to Homer1 (1:100) in blocking buffer for 2 days (44 h–52 h) at 4°C, followed by washing four times with blocking buffer, each time for 5 min with shaking.

49. Secondary antibody staining: Incubate sections with secondary anti-chicken donkey antibody conjugated with Alexa488 dye (1:200), anti-rat donkey antibody conjugated with Alexa568 dye (1:100), anti-mouse goat antibody conjugated with Atto647N dye (1:100), and anti-rabbit goat antibody conjugated with CF405M dye (1:100) in blocking buffer for 24 h at 4°C with shaking. Wash sections three times with blocking buffer, three times with high salt PBS, and three times with PBS, each time for 5 min with shaking.

Note: For the ExM, we changed the antibody concentration higher and the incubation time longer than usual immunostaining because fluorescent signals become weak in the expansion step.

ExM and Imaging

⏱ Timing: 4 days

ExM was performed according to a protocol published by Ed Boyden lab (Asano et al., 2018). For details on the ExM, please refer to http://expansionmicroscopy.org/.

50. Anchoring to gel: Incubate sections with Acryloyl-X (0.1 mg/mL) in PBS in 1.5-mL tube for more than 6 h at room temperature (22°C–26°C) with no shaking. Wash three times with PBS, each time for 5 min.

51. Infiltrate gelling solution: Incubate sections with the gelling solution in 1.5-mL tube for 30 min at 4°C in the dark.

Note: Remove PBS before anchoring and gelling by washing once with reagent. Gelling solution should be made just before use by mixing monomer solution, 4-hydroxy-TEMPO (inhibitor), APS, and TEMED (initiator, add last) and be kept at 4°C to prevent premature polymerization.

52. Gelation: Cut coverslip with a glasscutter and place two of them on the glass slide as spacers (Figures 4A and 4B). Drop 50 μL of gelling solution and transfer sections using paintbrush (Figures 4C and 4D). Slices are sandwiched between glass slide and top coverslip and incubate for 2 h at 37°C (Figures 4E and 4F).
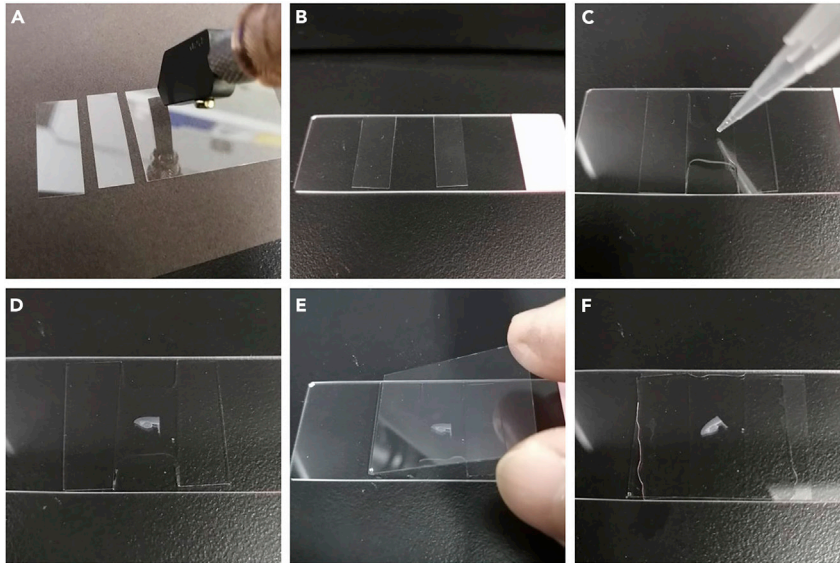
Figure 4. The Construction of the Gelling Chamber
(A) Cut coverslips with glasscutter.
(B) Place coverslips on the glass slide.
(C) Drop gelling buffer on the glass slide.
(D) Transfer sections into the buffer.
(E and F) Place a coverslip on the sample.

53. Digestion: Trim hippocampus regions minimally from gelled sections with a razor blade under a stereo microscope (Leica S9i) before detaching from the gel chambers (Figures 5A and 5B). Drop digestion buffer on the sample and peel off gently from the edge (Figures 5C–5E). Immerse sections in the 500 μL of digestion buffer at room temperature (22°C–26°C) for 12 h. Wash three times with PBS for 5 min.
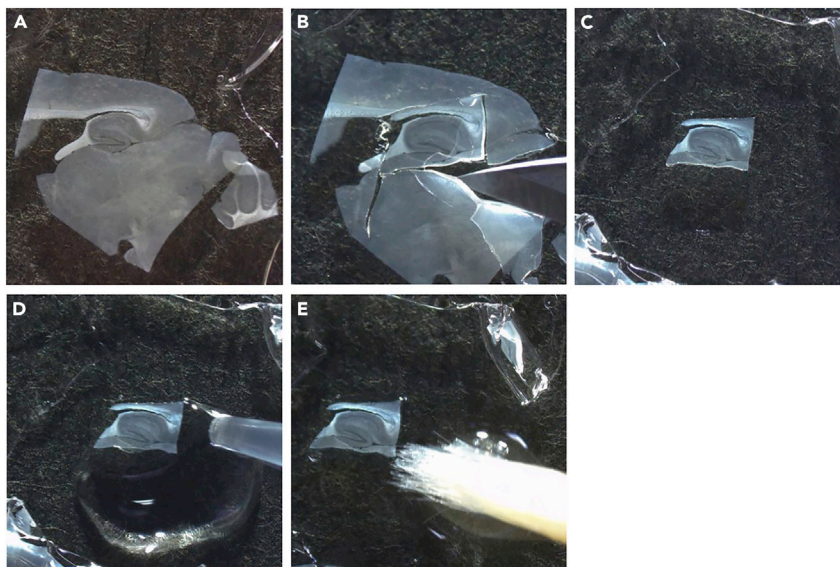
Figure 5. Detaching Gel of the Hippocampal Region from the Gelling Chamber
(A–C) Trim the hippocampal region with a razorblade.
(D) Drop digestion buffer on gel.
(E) Detach gel from the edge with a paintbrush.
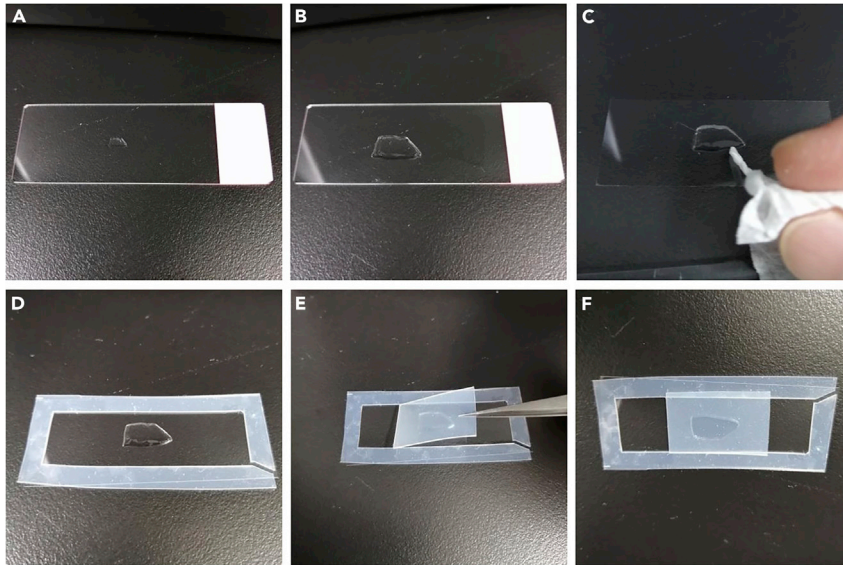
Figure 6. The Construction of the Imaging Chamber
(A and B) Comparison of the gels before and after expansion.
(C) Put expanded gel on the coverslip.
(D) Put silicon sheet surrounding the gel.
(E and F) Put silicon sheet on the sample.

> *Note:* Trimming the gel into an asymmetric shape is recommended to deduce the orientation easily. Be careful of losing a sample that becomes clear and invisible in the buffer after digestion. We recommend changing buffer by absorbing it with Kimwipes.

> ⫿⫿ **Pause Point:** Sample can be stored in PBS at 4°C in the dark until expansion.

54. Expansion: Transfer gels into new container that is enough size for expanded gels. Wash samples three times with water for 15 min to reach the final expansion size (×4 linearly, Figures 6A and 6B).

55. Imaging: Transfer samples on coverslip with paintbrush and remove extra buffer by Kimwipes. Silicon sheets (0.5-mm thick) are used as spacers surrounding the sample to prevent sample compression (Figures 6C and 6D). To avoid sample drifting during imaging, samples are sandwiched between a coverslip and silicon sheets (Figures 6E and 6F). Acquire fluorescence images using an inverted confocal fluorescent microscope (Olympus FV3000, using 10×/0.40 NA, 20×/0.70 NA, and 60×/1.20 NA water objective from Olympus). Use Imaris 9.1 (Bitplane) for generating three-dimensional (3D) volume rendering projection images or 3D maximum intensity projection images (Figures 7A and 7B).

> *Note:* Excess removal of surrounding water results in shrinking of the samples.

> ⫿⫿ **Pause Point:** After the imaging, samples can be stored in the shrunken state in PBS at 4°C in the dark.

EXPECTED OUTCOMES

Capturing images of the whole synaptic structures consisting of YFP-positive presynaptic terminals and mCherry-positive postsynaptic spines of excitatory neurons (Figures 7A and 7B) (also mCherry-positive presynaptic terminals and YFP-positive postsynaptic spines), and localization of Bassoon and Homer are simultaneously visualized in the synapses in the brain region which you are interested (Figures 8A–8E; in the hippocampus) (for example, hippocampus, cerebral cortex, etc.).
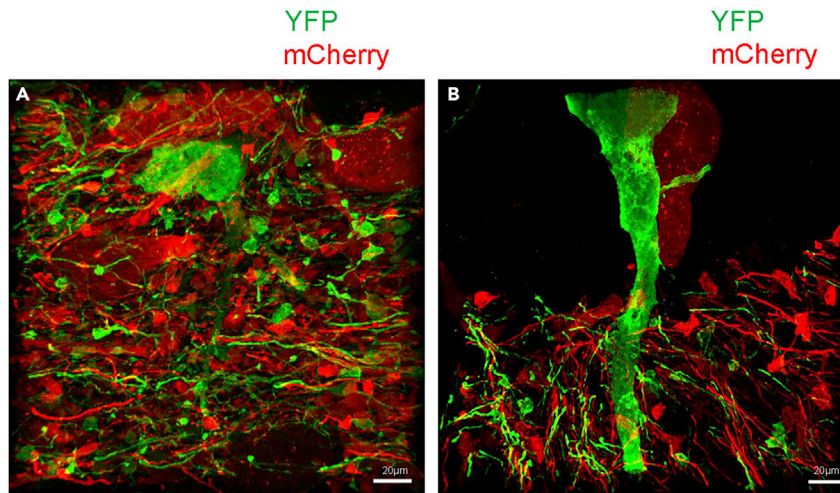
Figure 7. Representative Maximum Intensity Projection Three-Dimensional (3D) Images of the Hippocampus of BATTLE-1EX Slices
(A) Hilar mossy cells in the hippocampus.
(B) CA3 pyramidal cells in the hippocampus. Green and red show YFP and mCherry, respectively. Scale bar, 20 μm.

Neural circuits are connected and modulated by a considerable number of synapses. It has been an enormous challenge for many decades to develop a method for visualizing whole synaptic morphologies with simultaneous identification of endogenous synaptic proteins by light (Südhof and Malenka, 2008) (Arenkiel and Ehlers, 2009). The BATTLE-1 strategy and BATTLE-1EX method enabled the visualization of entire synaptic structures with endogenous synaptic proteins (Kohara et al., 2020). Lentivirus and AAV with serotype 9 used in the first version of BATTLE-1 are known to be infectable to the broad region of the brain. Furthermore, the CamKIIa promoter in the first version of BATTLE-1 is active in excitatory neurons in many brain areas (Benson et al., 1992). Therefore, researchers can apply BATTLE-1EX to the hippocampus or other regions. In future studies, AAV with different serotypes, such as the retrograde serotype and universal promoter, like actin promoter, might also be applied to the next-generation of BATTLE methods (Tervo et al., 2016). BATTLE-1EX may broadly contribute to research on synapses not only in fundamental neuroscience but also in disease neuroscience.

LIMITATIONS

In the current protocol, the BATTLE-recombinase systems could be applied to local regions of interest, such as the hippocampus (or cerebral cortex), but not brain-wide global regions.

TROUBLESHOOTING

Problem 1
Difficult to find synapses composed of YFP and mCherry-positive neurons (step 55).

Potential Solution
It is recommended to capture 3D image broadly by green and red colors using low-magnification objective lens first and use the 3D image as the quick overview for finding synapses. 60× water objective lens is recommended for high-resolution imaging because it is needed to switch the objective frequently to find synapses.

Problem 2
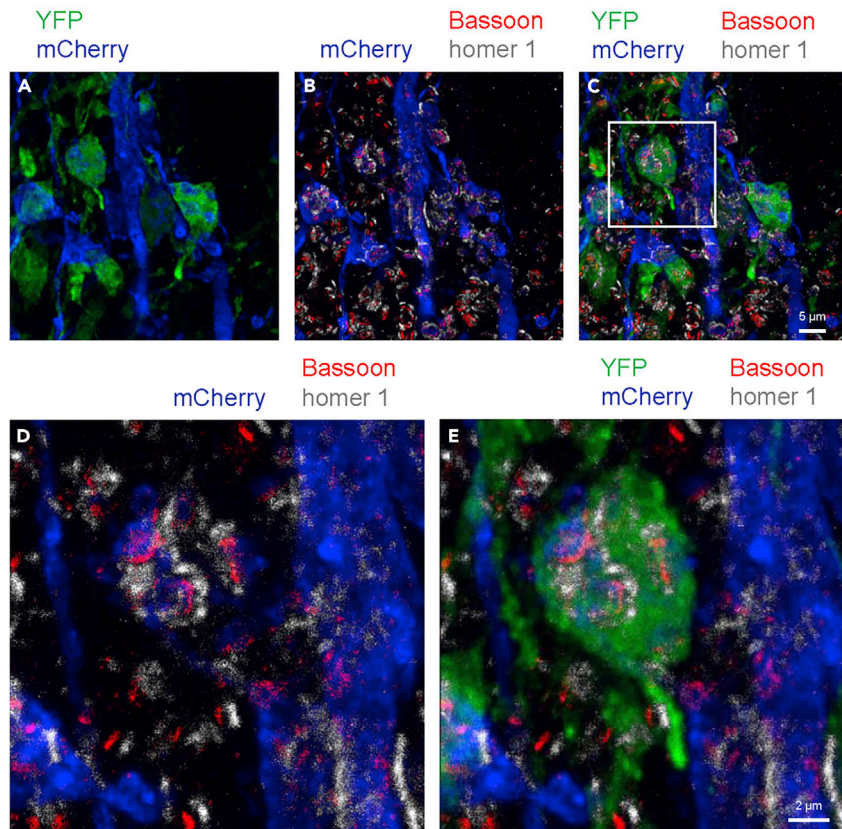Low fluorescence after expansion (steps 48 and 49).

Figure 8. Representative Maximum Intensity Projection 3D Image of the Synapse in the Hippocampus
(A–C) DG-CA3 synapses in the hippocampus. Scale bar, 5 μm.
(D) Enlarged image of box area in (B).
(E) Enlarged image of box area in (C). Scale bar, 2 μm. Blue, green, red, and white show YFP, mCherry, Homer, and Bassoon, respectively.

Potential Solution

We chose the fluorescent dye (Tillberg et al., 2016) because some fluorescent dyes such as cyanine family lose its signal during ExM. In addition, we prolonged incubation times of primary antibody for 2 days and of secondary antibody for 1 day to retain the signals after expansion. all antibodies excepting primary anti-GFP (chicken, 1:500) and secondary anti-chicken (Alexa488, 1:200) were used at a dilution of 1:100.

Problem 3

Photobleaching (step 55).

Potential Solution

In the fluorescent dyes used in this protocol, CF405M is subjected to photo bleaching during 3D imaging. To minimize the exposure time for the CF405M, we can adjust the imaging position using Alexa488, which maintains the intensity during the long imaging.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Keigo Kohara, Email: koharake@hirakata.kmu.ac.jp.

Materials Availability

All unique/stable reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and Code Availability

This study did not generate/analyze datasets and codes.

AUTHOR CONTRIBUTIONS

This protocol has been developed by K.K., A.I., H.H., T.K., and N.K. The detailed procedure was written by K.K., A.I., and N.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Arenkiel, B.R., and Ehlers, M.D. (2009). Molecular genetics and imaging technologies for circuit-based neuroanatomy. Nature 461, 900–907.

Asano, S.M., Gao, R., Wassie, A.T., Tillberg, P.W., Chen, F., and Boyden, E.S. (2018). Expansion Microscopy: protocols for imaging proteins and RNA in cells and tissues. Curr. Protoc. Cell Biol. 80, e56.

Benson, D.L., Isackson, P.J., Gall, C.M., and Jones, E.G. (1992). Contrasting patterns in the localization of glutamic acid decarboxylase and Ca2+/calmodulin protein kinase gene expression in the rat central nervous system. Neuroscience 46, 825–849.

Chen, F., Tillberg, P.W., and Boyden, E.S. (2015). Expansion microscopy. Science 347, 543–548.

Franklin, K., and Paxinos, G. (2008). The Mouse Brain in Stereotaxic Coordinates, Third Edition (Academic Press).

Livet, J., Weissman, T.A., Kang, H., Draft, R.W., Lu, J., Bennis, R.A., Sanes, J.R., and Lichtman, J.W.

(2007). Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. Nature 450, 56–62.

Luo, W., Mizuno, H., Iwata, R., Nakazawa, S., Yasuda, K., Itohara, S., and Iwasato, T. (2016). Supernova: A Versatile Vector System for Single-Cell Labeling and Gene Function Studies in vivo. Sci. Rep. 6, 35747.

Nimchinsky, E.A., Sabatini, B.L., and Svoboda, K. (2002). Structure and function of dendritic spines. Annu. Rev. Physiol. 64, 313–353.

Kohara, K., Pignatelli, M., Rivest, A.J., Jung, H.Y., Kitamura, T., Suh, J., Frank, D., Kajikawa, K., Mise, N., Obata, Y., et al. (2014). Cell type-specific genetic and optogenetic tools reveal hippocampal CA2 circuits. Nat. Neurosci. 17, 269–279.

Kohara, K., Inoue, A., Nakano, Y., Hirai, H., Kobayashi, T., Maruyama, M., Baba, R., and Kawashima, C. (2020). BATTLE: Genetically engineered strategies for split-tunable allocation of multiple transgenes in the nervous system. iScience 23, 101248.

Schlake, T., and Bode, J. (1994). Use of mutated FLP recognition target (FRT) sites for the exchange of expression cassettes at defined chromosomal loci. Biochemistry 33, 12746–12751.

Südhof, T.C., and Malenka, R.C. (2008). Understanding synapses: past, present, and future. Neuron 60, 469–476.

Tervo, D.G.R., Hwang, B., Viswanathan, S., Gaj, T., Lavzin, M., Ritola, K.D., Lindo, S., Michael, S., Kuleshova, E., Ojala, D., et al. (2016). A designer AAV variant permits efficient retrograde access to projection neurons. Neuron 92, 372–382.

Tillberg, P.W., Chen, F., Piatkevich, K.D., Zhao, Y., Yu, C.C., English, B.P., Gao, L., Martorell, A., Suk, H.J., Yoshida, F., et al. (2016). Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies. Nat. Biotechnol. 34, 987–992.

Torashima, T., Yamada, N., Itoh, M., Yamamoto, A., and Hirai, H. (2006). Exposure of lentiviral vectors to subneutral pH shifts the tropism from Purkinje cell to Bergmann glia. Eur. J. Neurosci. 24, 371–380.