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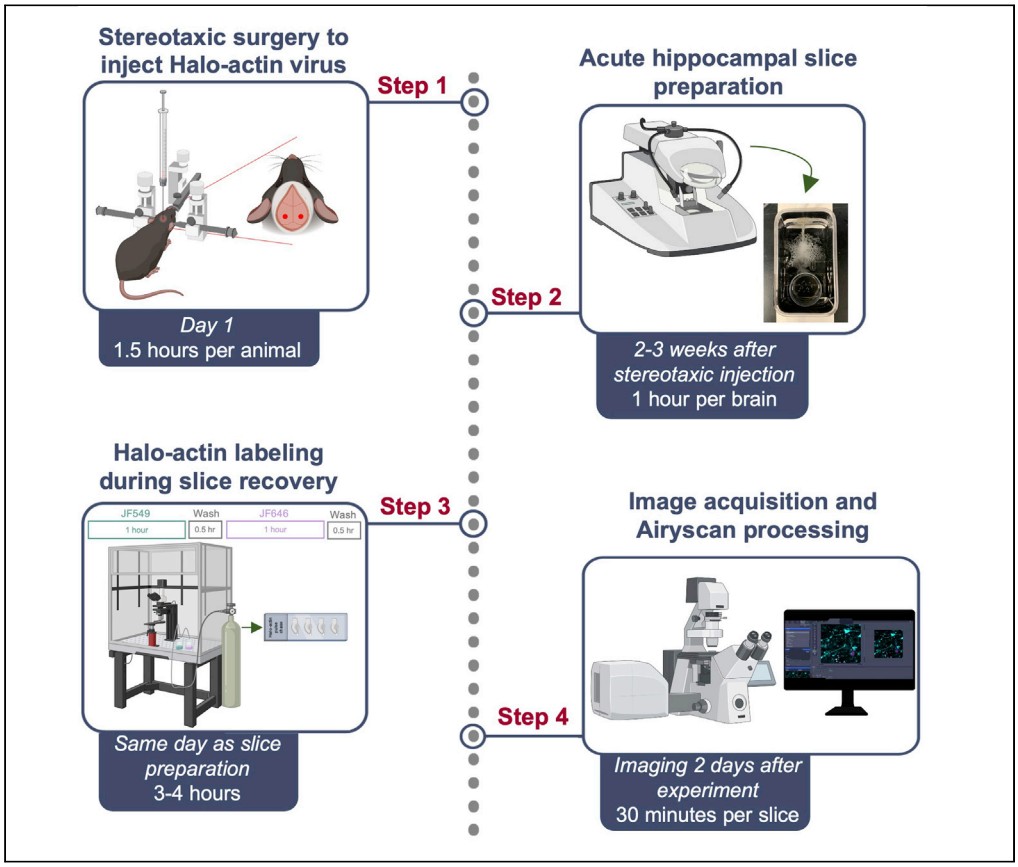
Protocol to study presynaptic protein synthesis in *ex vivo* mouse hippocampal slices using HaloTag technology



Presynaptic boutons in the mammalian brain are typically small and difficult to manipulate and study. Here, we present a protocol applying Halotag self-labeling technology to detect de novo local protein synthesis in intact presynaptic mossy fiber boutons from acute mouse hippocampal slices. We describe stereotaxic injection of Halotag-expressing virus into the brain region of interest, followed by brain slice preparation. We then detail the labeling of Halotag-fused protein and image acquisition to visualize the labeled protein in an intact circuit.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Stereotaxic injection of Halotag-fused gene of interest into brain region of interest

Preparation of acute hippocampal slices from adult mice (P35-45).

Halo-actin labeling to detect local newly synthesized actin in mossy fiber boutons

Imaging and Airyscan processing of brain slice images

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Protocol to study presynaptic protein synthesis in *ex vivo* mouse hippocampal slices using HaloTag technology

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SUMMARY

Presynaptic boutons in the mammalian brain are typically small and difficult to manipulate and study. Here, we present a protocol applying HaloTag self-labeling technology to detect de novo local protein synthesis in intact presynaptic mossy fiber boutons from acute mouse hippocampal slices. We describe stereotaxic injection of HaloTag-expressing virus into the brain region of interest, followed by brain slice preparation. We then detail the labeling of HaloTag-fused protein and image acquisition to visualize the labeled protein in an intact circuit. For complete details on the use and execution of this protocol, please refer to Monday et al. (2022).[1]

BEFORE YOU BEGIN

Understanding the detailed molecular translation events that support synaptic transmission requires high-resolution methods that provide quantitative information and molecular specificity. We developed the following protocol to assess direct evidence of presynaptic translation in acute brain slices. Individual proteins can be tagged using the HaloTag system to monitor them. Using a pulse-chase assay, we demonstrated local protein synthesis where we were able to visualize newly synthesized β-actin in mossy fiber boutons following axonal transection, wherein the cell body layer was manually separated (using a blade) from its axonal projections to prevent any protein trafficking, as well as after neuronal activity.

Self-labeling tags such as the HaloTag allow covalent labeling of a protein of interest with organic dyes. These dyes are far brighter and more photostable than conventional fluorescent proteins and compatible with tissue imaging. Development of fluorescent and cell-permeable dyes at the Janelia Research Campus (JF dyes) have been instrumental in our ability to see inside of tissue. Furthermore, self-labeling systems afford flexibility in performing pulse-chase, dual-labeling or photoactivation assays to quantify differences in fluorescence signal intensity, size and location over time.

Hippocampal mossy fiber (MF) axons extend from the granule cells of the dentate gyrus (DG) and project through the stratum lucidum to the area CA3 region, where they synapse onto CA3 pyramidal cells (MF-CA3) and interneurons.[2–4] As the primary excitatory drive into the hippocampus proper, MFs are critical for hippocampus-dependent forms of learning and memory. To investigate the role of local translation in presynaptic plasticity, we took advantage of mossy fiber bouton (MFB) terminals as a model system. These boutons are exceptionally large (4–8 μm diameter, much larger than a dendritic spine) and can dynamically change in structure in response to experiences and

undergo presynaptic forms of plasticity—which make MFBs ideal for combined electrophysiological and imaging methods. The dentate gyrus and the hippocampus proper form two offset C-shaped rings that interlock. This unique structural feature allows for experimental manipulation of the dentate gyrus cell body layer, such as targeted viral injections to a specific population of neurons, as well as transections to physically sever the cell bodies from their axons.

Lastly, it is important to consider the design of the reporter. For example, it is necessary to identify whether the protein of interest is expressed in the dentate gyrus granule cells and whether it is present in mature MFBs. Next, determine which end of the protein the fluorescence tag should be fused to. Also, if the protein is presumed to be locally translated, it is recommended to include the 3′UTR to the reporter to facilitate efficient mRNA trafficking to axons.

Here, we describe a protocol that allows for substantial improvement in the imaging of presynaptic boutons to measure quantitative differences in translational activity in brain tissue.

Institutional permissions

Mice of both sexes were group housed in a standard 12-h light/12-h dark cycle. Experimental procedures adhered to NIH and Albert Einstein College of Medicine Institutional Animal Care and Use Committee guidelines. Mice are group housed before and after stereotaxic surgery (protocol number 00001043).

Plasmid preparation and lentivirus production

Timing: 4–8 weeks (for step 1)

In our study, we used HaloTag fused to β-actin followed by the 3′UTR to detect newly synthesized Halo-actin in MFBs[1] (previously in hippocampal neuronal culture[5]). Bath application of ultrabright membrane permeable JF dyes conjugated to the HaloTag ligand (JF-HTL) allows for the labeling of the HaloTag fusion proteins with very high affinity and specificity.[6]

If your protein of interest is not available on Addgene, knowledge of cloning will be necessary. It is also important to note that creating a new plasmid will increase time needed for preparation.

Additionally, it is absolutely necessary to obtain high-titer lentiviruses.

⚠ CRITICAL: Stereotaxic surgery setup and vibratome are needed for successful completion of this protocol.

1. Order the Halo-actin reporter plasmid from Addgene (Addgene_102718) and perform Midiprep of the lentivirus expression vector (protocol found in downloads on website: https://www.mn-net.com/us/nucleobond-xtra-midi-kit-for-transfection-grade-plasmid-dna-740410.50).

⚠ CRITICAL: To ensure accuracy, lentivirus expression vector should be sequenced before proceeding.

2. Concentrate lentivirus.
   a. Seed a 15-cm petri dish with HEK293T cells and grow to 70% confluence.
   b. Transfect cells using calcium phosphate.
   c. After 48 and 72 h, collect media and filter (0.22 μm).
   d. To obtain high titer lentivirus, centrifuge at 19000 RPM (65,000 × *g*) for 2 h and resuspend virus. To measure final lentiviral titer, use a qRT-PCR titration kit.

⚠ CRITICAL: Viral titer should be equal to or higher than 10^{11} particles/mL. When viral concentrations are lower than indicated, it is difficult to achieve expression in dentate granule cells of the hippocampus.

⚠ CRITICAL: For the purposes of this protocol, use of lentivirus allows sparse labeling of dentate granule cells, which is ideal for high resolution imaging of individual boutons. If your usage does not require sparse labeling, Halo-actin can also be used in a recombinant AAV vector.

⚠ CRITICAL: Lentivirus (BSL-2) must be transported on dry ice and remain in −80°C freezer.

3. Order Janelia Fluor® HaloTag® Ligands from Promega. For the purposes of this protocol, we utilized Janelia Fluor 549-HaloTag ligand and Janelia Fluor 646-HaloTag ligand.

Note: HaloTag-ligand conjugated to a tetraalkylrhodamine derivatives (JF549-HTL and JF646-HTL) should be stored at or below −30°C. We prepared a stock solution of each dye at 100 μM in dimethyl sulfoxide (DMSO). We store our dyes at −20°C for up to 1 month after aliquoting. For our experiments, we use a final concentration of 100 nM of JF549-HTL and 200 nM of JF646-HTL.

Acquire wild-type P21-P24 mice

Timing: 1 week - continuous

For the purposes of this protocol, we stereotaxically injected both male and female WT C57BL/6J aged P21-P24.

4. Acquire P21 mice from Charles River (Cat# 027) or from your in-house breeding colony.

⚠ CRITICAL: If mice are delivered from Charles River or another source, it is important not to perform stereotaxic surgery on the same day and let the mice recover, at least overnight (10–12 h). Stress from the move may result in death during surgery.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Primary Antibody HaloTag antibody (1:500)	Promega Corp.	G9281, RRID: AB_713650
Chemicals, peptides, and recombinant proteins		
Cycloheximide (CHX)	Tocris Bioscience	Cat#0970
Anisomycin	ALFA Aesar	CAS No. 22862-76-6
PBS, phosphate buffered saline, 10× Solution	Gibco	Ref. 70011-044
Paraformaldehyde	Electron Microscopy Sciences	Cat#15710
NaCl	Sigma-Aldrich	Cat# 7647-14-5
N-Methyl-d-glucamin (NMDG)	Sigma-Aldrich	Cat# 6284-40-8
KCl	Sigma-Aldrich	Cat# 7447-40-7
$NaHCO_3$	Sigma-Aldrich	Cat# 144-55-8
NaH_2PO_4	Sigma-Aldrich	Cat# 10049-21-5
$CaCl_2$	Sigma-Aldrich	Cat# 10035-04-8
$MgSO_4$	Sigma-Aldrich	Cat# 10034-99-8
D-Glucose	Sigma-Aldrich	Cat# 50-99-7

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
HEPES	Sigma-Aldrich	Cat# 7365-45-9
Thiourea	Sigma-Aldrich	Cat# 62-56-6
Na-Ascorbate	Sigma-Aldrich	Cat# 134-03-2
Na-Pyruvate	Sigma-Aldrich	Cat# 113-24-6
$MgCl_2$	Sigma-Aldrich	Cat# 7791-18-6
Agar	Sigma-Aldrich	Cat# 9002-18-0
Janelia Fluor® 549-HaloTag® ligand	Promega Corp.	Cat# GA1110
Janelia Fluor® 646-HaloTag® ligand	Promega Corp.	Cat# GA1120
Photoactivatable-Janelia Fluor 646	Janelia Research Campus, Ashburn, VA, USA	N/A
ProLong™ Diamond Antifade Mountant with DAPI	Invitrogen	Cat# p36962
Dimethyl sulfoxide (DMSO)	Millipore	CAS# 67-68-5
Critical commercial assays		
Nucleobond Xtra Midi kit	Macherey-Nagel	740410.100
Lenti-X™ qRT-PCR Titration Kit	Takara	Cat#631235
Experimental models: Organisms/strains		
P21 mice: C57BL/6NCrl (males and females)	Charles River	Cat# 027; RRID:IMSR_CRL:027
Experimental models: Cell lines		
HEK293T cells (no more than 12 passages)	Thermo Fisher	Cat# R70007
Recombinant DNA		
HaloTag-bActinCDS-bActinUTR-MS2V5 (Halo-actin reporter)	Singer Lab, and previously in (Yoon et al.)[5]	Addgene plasmid #102718; RRID: Addgene_102718
Gen3 lentivirus packaging plasmid (pMD2.G) (VSVG)	Singer Lab, and previously in (Yoon et al.)[5]	Addgene plasmid #12259; RRID: Addgene_12259
Gen3 lentivirus packaging plasmid (pMDLg/pRRE)	Singer Lab, and previously in (Yoon et al.)[5]	Addgene plasmid #12251; RRID: Addgene_12251
Gen3 lentivirus packaging plasmid (pRSV-Rev)	Singer Lab, and previously in (Yoon et al.)[5]	Addgene plasmid #12253; RRID: Addgene_12253
Software and algorithms		
Graphpad Prism 8	Graphpad	https://www.graphpad.com/scientific-software/prism/
ImageJ FIJI	Schindelin et al.[7]	http://imagej.net/Welcome
Other		
Hamilton syringe	Hamilton	80075
PrecisionGlideTM Needle 27G	BD	Ref. 305109
Poly-lined towel/drape	Henry Schein	Ref. 900-4686
Brain infusion pump	KOPF	940090A
Stereotax	KOPF	Model 940
Heat lamp	Amazon	B01E9IY6US
Heating pad	Amazon	B00075M1T6
Ideal Micro-Drill	CellPoint Scientific	5474
20 mL granulated cylinders	Corning	No. 1000
Vibratome	Leica	1200S
Fisherbrand™ Premium Superfrost™ Microscope Slides	Fisher Scientific	Cat#12-544-3
Corning cover glass No. 1.5 22 mm sq	Corning	Cat#2870-22
Kimwipes	KimTech	Cat#34155
Paintbrush for hippocampal brain slices: pointed tip paint brush	Amazon	B07PYQH1FS
Multiwell plate with lids	CytoOne	CC7672-7524
VAPRO Vapor Pressure Osmometer	ELITechGroup	Model 5600
Compressed gas (5% carbon dioxide, 95% oxygen)	Airgas	N/A
Bead sterilizer	CellPoint Scientific	GERMINATOR 500™

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Isoflurane	Covetrus	029405
Isoflurane vaporizer – funnel-fill	VetEquip	Item # 911103
Ophthalmic solution	Dechra	NDC 17033-211-38
Suture, Nylon, 5-0, NFS-2	Fisher Scientific (Oasis)	501180630
6″ Cotton tipped wood applicator	Dynarex	4305
Confocal microscope	ZEISS Research Microscopy Solutions	Zeiss LSM 880 Airyscan

MATERIALS AND EQUIPMENT

All essential buffers and solutions should be freshly made prior to sample preparation and used within 3 days to avoid degradation or loss of efficacy. Make sure there is enough of all solutions before starting that are required for slice preparation, incubation, HaloTag labeling, fixation and mounting. Please follow storage instructions as indicated on each item.

NMDG solution

Older animals (>P28) are more susceptible to brain damage due to excitotoxicity during slicing. For this reason, we utilized N-methyl-D-glucamine (NMDG), which has been shown to be protective by directly reducing transient depolarization-activated outward currents.[8,9]

Start with a 2 L graduated cylinder and fill up with Milli-Q water to about 1.8 L and then slowly measure and add compounds indicated below.

Compounds	Final concentration (in mM)	Amount in g (for 2 L total)
NMDG	93	36.3090
KCl	2.5	0.3778
NaH_2PO_4	1.25	0.3000
$NaHCO_3$	30	5.0404
HEPES	20	9.5320
Glucose	25	9.0078
Sodium ascorbate	5	1.9810
Thiourea	2	0.3044
Sodium pyruvate	3	0.6600
$MgCl_2$	10	1.9042
$CaCl_2$	0.5	0.1470
Sterile Milli-Q water		fill to 2 L
Total		**2 L**

After the solution has been stirring for some time, pH should be measured (should be around pH 8). Adjust pH to 7.3–7.4 by adding concentrated HCl (about 5 µL at a time).

⚠ CRITICAL: Concentrated HCl should always be handled with extreme care. To avoid chemical burns, user should wear gloves when handling while under chemical hood.

Storage note: Extra NMDG can be aliquoted and frozen in −20°C and can be used for up to one month. If thawed, however, it should be used within 3 days to avoid degradation or loss of efficacy (Expect to use about 300 mL of NMDG per mouse).

ACSF (Artificial CerebroSpinal fluid) solution

Start with a 4 L graduated cylinder and fill up with Milli-Q water to about 3.8 L and add compounds indicated below.

Compounds	Final concentration (in mM)	Amount in g (for 4 L total)
NaCl	124	28.98624
$NaHCO_3$	26	8.73704
Glucose	10	7.2064
KCl	2.5	0.7455
NaH_2PO_4	1	0.55196
$MgSO_4$	1.3	1.2818
$CaCl_2$	2.5	1.47
Sterile Milli-Q water		fill to 4 L
Total		**4 L**

While solution stirs, osmometer should be calibrated using Opti-Mole osmolality standards. After the solution has been stirring for some time, measure osmolality (Desired range 297–303 mmol/kg).

Storage note: ACSF can be stored at 4°C for up to 3 days.

Phosphate-Buffered Saline (PBS)		
Reagent	Final concentration	Amount
10× PBS	1×	5 mL
Sterile Milli-Q water		Up to 50 mL in falcon tube
Storage note: PBS can be stored at 4°C for up to 7 days.		

Paraformaldehyde solution (PFA)		
Reagent	Final concentration	Amount
Paraformaldehyde (16%) Solution	4%	10 mL
10× PBS	1×	4 mL
Sterile Milli-Q water		Up to 40 mL in falcon tube

⚠ CRITICAL: PFA is a confirmed human carcinogen and irritates the skin, eyes, and respiratory tract. Prolonged exposure can have harmful effects. Prepare this solution under a chemical hood with gloves and dispose of it in an appropriate biohazard waste container.

Storage note: PFA solution should be stored at 4°C for no more than 7 days.

0.9% Saline solution		
Reagent	Final concentration	Amount
NaCl	0.9%	0.45 g
Sterile Milli-Q water		Up to 50 mL in falcon tube
Storage note: Saline solution should be stored at room temperature (RT) for use during surgery for no more than 2 weeks.		

Agar for block		
Reagent	Final concentration	Amount
Agar	4%	8.8 g
Sterile Milli-Q water		220 mL
Add stir bar, and heat until bubbling. Pour into mold (pictured in Figure 1, an inverted pipette tip box lid with holes for cotton tip wood applicators which when cut, create wells for the hippocampi during vibratome slicing).		

STEP-BY-STEP METHOD DETAILS

Stereotaxic surgery

Timing: 1.5 h per animal

A targeted stereotaxic injection straight into the dentate gyrus of anesthetized mice allows for site specific expression of Halo-actin in the soma and distant boutons of dentate granule cells.

Please adjust injection protocols in accordance with Institutional Animal Care and Use Committee regulations as well as Institutional Environmental Health and Biological Safety requirements. In our case, all experimental procedures were approved by Albert Einstein College of Medicine's Institutional Animal Care and Use Committee (protocol numbers 00001043 and 00001053).

1. Prepare Hamilton syringe. Disinfect with 70% ethanol and rinse with Milli-Q water multiple times.

⚠ CRITICAL: Ensure no ethanol remains in the Hamilton syringe before virus is loaded. This can lead to viral loss and no expression in injected animals.

⚠ CRITICAL: The method for disinfecting lentiviruses is to soak in a 10% bleach solution for several minutes followed by rinse in water and disposal into medical waste bins.

2. Disinfect and prepare stereotaxic surgical setup and tools.
   a. This can be achieved by wiping setup and all tools with 70% ethanol and Kimwipe. It is also recommended to disinfect tools in bead sterilizer prior to usage.
3. While tools are in bead sterilizer, pickup animals from animal facility. It is recommended to bring all animals to a bench near your stereotaxic setup to have consistency in time between animals.
4. Prepare dental drill and surgical supplies in surgical area.
   a. Obtain betadine, premixed 0.9% saline solution, and 70% ethanol in 50 mL falcon tubes. Prepare small container with 10% bleach.
   b. Fill the vaporizer with isoflurane. Ensure oxygen is connected well and the general anesthesia setup is functional.
   c. Place all sterilized tools on sterile pad.
   d. Gather ophthalmic ointment.
   e. Prepare microinjector pump control.
   f. Thaw −80°C Halo-actin reporter viral aliquots by placing them on wet ice.
   g. Once Hamilton syringe is attached to stereotaxic setup according to manufacturer's instructions, slowly load virus into the syringe. Load enough virus to inject all mice for the day plus 20% extra.

Note: Viral stock is susceptible to titer loss from freeze-thaw cycles. To ensure consistent titers, remove aliquots from −80°C freezer as needed.

5. Prepare recovery area.
   a. Gently place a clean cage with top on a heating pad.

⚠ CRITICAL: Ensure that cage has no bedding. This can lead to choking as animal wakes up from anesthesia.

6. Anesthetize and mount mouse on stereotaxic apparatus.
   a. Turn on oxygen flow to the isoflurane induction chamber.

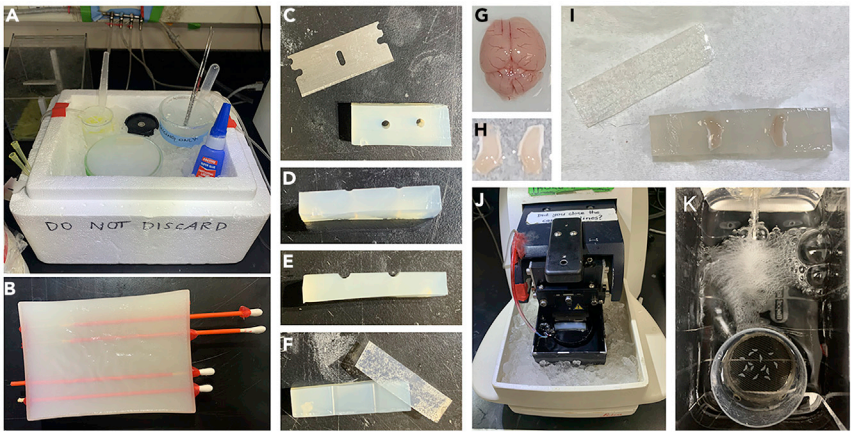

Figure 1. Schematic of slice preparation for hippocampi isolation

(A) Organization of tools and equipment for hippocampi isolation.
(B) Agar block before cutting to appropriate size for specific purposes.
(C) Representative agar block with wells.
(D) Agar block that has been cut at a slight angle allowing for one side for dorsal hippocampus.
(E) Agar block that has been cut at a slight angle allowing for one side for ventral hippocampus.
(F) Small slice from bottom of agar block cut to act as lid.
(G) Brain isolation.
(H) Hippocampal dissection.
(I) Hippocampi placed in wells in agar block.
(J) Vibratome setup.
(K) Slices in bubbling ACSF-filled slice holding chamber which contains a plastic mesh fixed on a ring.

b. Place the mouse in the induction chamber and then turn on flow of isoflurane to 5%. Wait for the mouse to become sedated. After initial rapid breathing, breathing normalization (a single breath every 3 s is indicative of sufficient sedation).
c. Adjust flow from induction chamber and decrease from 5% to 3% isoflurane.
d. Gently remove mouse from chamber and mount onto the stereotaxic frame using bite bar.
e. Slide nose cone over mouse's nose, then decrease the flow of isoflurane to 2%.

Note: Pinching the hind paw is another way to ensure proper sedation has been achieved before securing animal in the stereotaxic frame.

⚠ CRITICAL: It is vital to adjust level of anesthesia according to mouse breathing rate and sedation. Faster respiration may require slow increases in anesthesia and should be adjusted accordingly throughout the experimental procedure.

f. Gently adjust the mouse so that mouse head looks straight and level.
g. Gently progress the ear bars into the ear canal. In conjunction with the nose cone, the skull should be completely stabilized.
h. If there is still movement, loosen ear bars, adjust the mouse head and try again.
i. To test whether the skull is stable, look at the head from top and then from the side to ensure it looks level. Press gently on top of head to make sure mouse does not move in response.
j. Place ophthalmic ointment on mouse eyes using sterile swab.

7. Expose the skull.
   a. Gently shave the fur from the mouse head.
   b. Sterilize the scalp by applying betadine on a cotton swab in circular motion.
   c. Using surgical scissors, make a single vertical incision at the midline starting from the posterior to the eyes.

d. Splay each layer of tissue open until you have a visible field of view.
e. Using a sterile swab with 70% EtOH, gently wipe the exposed skull. This cleans off any muscle and tissue still attached before drilling.

8. Inject lentivirus into the region of interest.
   a. Determine injection location using dissection scope or magnification glasses.
      i. Firstly, find bregma.
      ii. For lentivirus injection into the DG between P21-P24, established coordinates are -2.2 posterior to Bregma (y-coordinate), 2.0 laterally (x-coordinate), and 2.0 ventral (z-coordinate) from dura with lambda being 0.05 units higher than bregma.[10]
   b. After determining x- and y-coordinates, lower tip to the skull where it is barely touching and zero the microinjector pump control at bregma.
   c. Then, go to lambda and measure z-coordinate.
   d. Adjust nose cone height until you achieve an angle of 0.05.
   e. After value is recorded, raise syringe to avoid drill hitting the tip.
   f. Using micro-drill, slowly drill a single bore hole through the skill at point of determined injection at x-and y-position.
      i. Slight bleeding is normal, but care should be taken to pause until bleeding stops.
   g. Before lowering needle into brain through drilled burr hole, manually press button on microinjector and continue to apply pressure until you see a bead of liquid exit the tip of the Hamilton syringe.
   h. Using paper tissue (Kimwipe), gently wipe away the bead of the injection solution and discharge into a small container of 10% bleach.
   i. Inject viral solution.
      i. Re-position the tip of the injection syringe to the brain's surface. Gently advance until you go 2.0 ventral from dura.
      ii. Eject virus at a flowrate with a total volume of 1.5 mL/ hemisphere at a flow rate of 0.2 mL/ min.
   j. Do not withdraw the needle right after injection. Wait at least 7–8 min to allow injection solution to spread locally into tissue. After sufficient time has passed, very gently withdraw the needle over 1–2 min.
9. Close the scalp.
   a. Using tweezers, pull together two halves of the scalp and suture (Nylon, 5-0, NFS-2).
10. Remove ear bars and deliver a small injection of normal saline for post-surgical fluid resuscitation (1 mL). Place mouse in the recovery cage with heated pad underneath.
11. Clean up the surgical area, disinfect and dispose of all material appropriately. Repeat steps 1–10 for all remaining experimental cohort.

Note: We do not wash the syringe between injections, as exposure of the tip to 70% ethanol will result in viral loss. In between injections, the syringe is stored within a secondary container.

Note: Follow all institutional guidelines for post-surgical care.

Note: In order to achieve optimal expression of your reporter or biosensor, we strongly recommend testing different amounts of virus, as well as flowrates. Duration of expression should also be optimized. For the purposes of this protocol, we waited for 2–3 weeks after injection for expression.

Acute hippocampal slice preparation

Timing: 1 h

These steps will result in preparation of acute hippocampal slices from virus-injected mouse (age range: P35-45).

12. Gather tools and solutions for hippocampal dissection and slicing. Prepare ice bucket (Figure 1A).
    a. Prepare NMDG solution. Prepare ACSF. Bubble NMDG and ACSF with carbogen (5% carbon dioxide, 95% oxygen) for at least 10 min on ice before starting.
    b. Remove agar from fridge (that was prepared in mold) and cut block with blade (Figures 1B–1F). Cut perpendicular to create a block (Figure 1C), cut across to create a well (Figures 1D and 1E), and then thinly slice bottom to create a lid (Figure 1F).
    c. Calibrate vibratome blade and set proper settings.
13. Expose the experimental animal to isoflurane until the nociceptive reflexes expire.
14. In under 30 s, decapitate the animal, remove brain (Figure 1G) and put into ice cold NMDG cutting solution.
15. Isolate hippocampi (Figure 1H), adjust on block (Figure 1I), and cut using VT1200s vibratome at a thickness of 300 μm (recommended cutting speed: 0.1 mm/s, Figure 1J). Fill the slicing chamber with ice-cold NMDG and fill the outer chamber of the vibratome with ice.

⚠ CRITICAL: The MF axons are long and fragile, so it is important that the hippocampal dissection is done extremely carefully, and hippocampi are placed securely in the block to avoid damaging the mossy fibers during slicing.

Note: Alternative slice orientations to study other brain regions should work equally well. This particular set of instructions will result in transverse hippocampal slices, and coronal slices can be cut as well. All subsequent steps in this protocol will remain nearly identical regardless of the brain region.

16. Use a transfer pipette to move slices into the bubbling ACSF-filled slice holding chamber which contains a plastic mesh fixed onto a ring[11] (Figure 1K).

Pause point: Let the slices recover for at least 30 min in the slice holding chamber at room temperature (RT, 21°C–24°C) before beginning HaloTag labeling protocol. Make sure the brain slices stay continuously oxygenated.

Halo-actin labeling during slice recovery

Timing: 3–4 h

This major step describes the pulse-chase assay which allows us to label Halo-actin that was newly synthesized in an intervening time window. This approach enables the detection of newly synthesized Halo-actin in MFBs.

17. While slices recover, remove the dye-conjugated HTL of choice from the −20°C freezer.
    a. Prepare a desired dilution of HTL in ACSF from stock solution in a 20 mL Pyrex beaker.

Note: The final concentration of JF549-HTL is 100 nM and JF646-HTL is 200 nM. The labeling conditions may need to be determined empirically for your specific gene of interest, using appropriate negative control experiments such as cycloheximide (CHX, translation inhibitor).

18. For the detection of newly translated proteins, cell-permeable Halo-ligand conjugated to a tetraalkylrhodamine derivatives (JF549-HTL or JF646-HTL) will be bath-applied sequentially for the pulse-chase assay.
    a. Using 20 mL Pyrex beaker, add RT ACSF and start bubbling with carbogen and add JF549-HTL to a final concentration of 100 nM.
    b. After 10 min of bubbling, gently collect slices with a transfer pipette and place in the beaker.
    c. Label for 1 h. 10 min before the hour is up, obtain a second 20 mL beaker and start bubbling RT ACSF (10 mL).

d. After the hour is up, gently remove slices from the dye cocktail picking up as little of solution as possible and transfer to bubbling ACSF.
e. Wash in ACSF for 30 min. 10 min before the 30 min are up, obtain another graduated cylinder and start bubbling RT ACSF.

Pause point: Bath-application of halo dye and wash out will now allow you to detect Halo-actin expressing cells using epifluorescence to screen slices. If your experimental question requires detecting newly synthesized protein after a certain manipulation, pre-screening slices for detectable viral expression is advised.

⚠ CRITICAL: Slices must stay well oxygenated throughout all procedures.

f. Towards the end of the 30-min ACSF wash or experimental manipulations (if performed), add JF646-HTL (200 nM) to the bubbling ACSF beaker and pipette slices into this beaker to label newly synthesized Halo-actin. Label for 1 h.
g. Finally, transfer labeled slices to a fresh ACSF beaker and wash out for 30 min.

Note: Some controls that can be conducted here include reversing order of dye and adding translation inhibitor, CHX (80 μM).

19. After washout, fix slices with 4% PFA in a 24-well plate overnight (10–12 h) at 4°C. Wrap the plate with aluminum foil to avoid any bleaching of fluorescence signal.
20. Next day, replace PFA with 1× PBS.
21. Mount and arrange the sections without any folds or wrinkles onto slides using a brush. Take extreme care to not damage the tissue sections.
    a. Multiple hippocampal slices can be mounted on the same slide.
    b. Remove excess 1× PBS with Kimwipe.
22. Apply few drops of ProLong™ Diamond Antifade Mountant with DAPI and place cover glass.
23. Leave the slides in the dark at RT and let them cure overnight (10–12 h). Seal the slides the following day with nail polish, let dry, and store slides at 4°C.

Image acquisition and Airyscan processing

Timing: 30 min per hippocampal slice

MFBs are highly variable in shape and size.[12] To achieve high-resolution images of these boutons, and to be able to precisely detect changes in Halo-actin synthesis, we turned to Zeiss LSM 880 with Airyscan. A classic confocal microscope utilizes a pinhole to reject out-of-focus light from reaching the detector to generate an optically sectioned image. Although in principle a small pinhole improves optical resolution, in most biological imaging a compromise must be made between image resolution and signal-to-noise. To overcome limitations of traditional scanning confocal microscopy, Zeiss introduced the Airyscan detector, which is composed of a 32-channel area detector set in a shape of a honeycomb, where each channel simultaneously captures a different position of the point spread function. This allows each channel to behave as a small pinhole to maximize resolution while allowing a larger overall pinhole to improve signal-to-noise ratio.[13]

24. On the day of imaging, turn on the microscope and necessary lasers (561 nm and 647 nm laser lines).
    a. Clean the 63× objective.
    b. Settings as follows:
        i. Plan-Apochromat 63×/1.4 Oil DIC M27 and 1.8× zoom.
        ii. Keep threshold, laser power, and gain constant for each experimental group imaged.
        iii. Also, importantly, set pixel width and height as 0.049 μm and voxel depth as 0.187 μm.

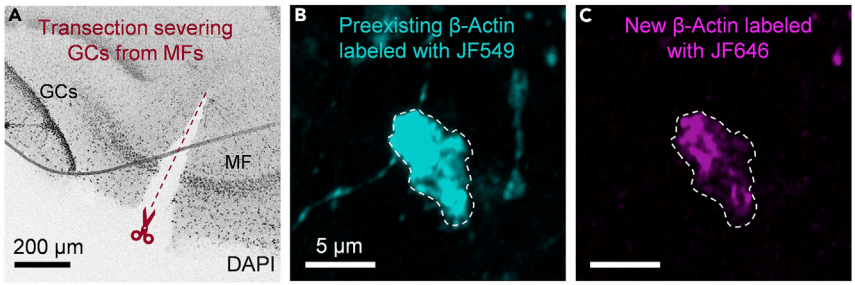

Figure 2. Expected outcome of transection experiment

(A) Representative fluorescence image of DAPI-stained hippocampal slice to show the cut severing GC somas from MF axons acquired at 10× magnification.

(B and C) Transected MF axons resulting in Halo-actin synthesis in MFBs acquired at 63× magnification. Please refer to Monday and Kharod et al.[1] for entire experiment.

25. After locating the stratum lucidum, take z-stacks throughout axon bundle at similar depths such that boutons are captured in their entirety.
    a. Make sure step size is set to optimal.

Note: For our purposes, we took three z-stacks through the entire z-plane of the tissue where boutons are visible (one in CA3a, one in CA3b, and one in CA3c). At the end, the depth imaged was about 10 µm with a z-stack consisting of about 50 frames.

26. Airyscan process images. Make sure acquisition settings remain identical between treatment groups and imaging days.

EXPECTED OUTCOMES

This protocol can be used to investigate how your protein of interest is synthesized locally in dendritic spines and presynaptic terminals.

For our purposes, we hypothesized that our candidate protein, β-actin, may be synthesized locally in presynaptic mMFBs. To test this possibility, we transected slices, making a small cut perpendicular to stratum lucidum to sever GC somata from the MF axon to prevent any trafficking of proteins from the GC soma to the distant boutons (Figure 2A), which was followed by a sequential labeling of Halo-actin. We found that MFBs are able to synthesize Halo-actin at the same level as non-transected slices (Figures 2B and 2C). The pulse-chase assay using self-labeling HaloTag allows us to label preexisting Halo-actin with one color and then subsequently label only newly synthesized Halo-actin with another color.

Functionally, long-term synaptic plasticity is defined by long-lasting activity dependent changes in synaptic efficacy and presynaptic long-term plasticity (LTP) is associated with changes in transmitter release and structure of the presynaptic terminal. We sought to determine whether presynaptic MF-LTP is also associated with local protein synthesis. We found that delivering a MF-LTP induction protocol (Figure 3A) resulted in an increase in Halo-actin levels (Figures 3B and 3C), indicating that MF-LTP is associated with an increase in local synthesis of β-actin in distant boutons. To induce MF-LTP, two borosilicate glass stimulating pipettes filled with ACSF were placed in the GC layer of the dentate gyrus at the border of the hilus. The LTP induction protocol consists of 125 pulses, 25 Hz, repeated 3 times every 20 s.

QUANTIFICATION AND STATISTICAL ANALYSIS

Fluorescence intensity measurements for HaloTag-labeled signal

Timing: 1 day

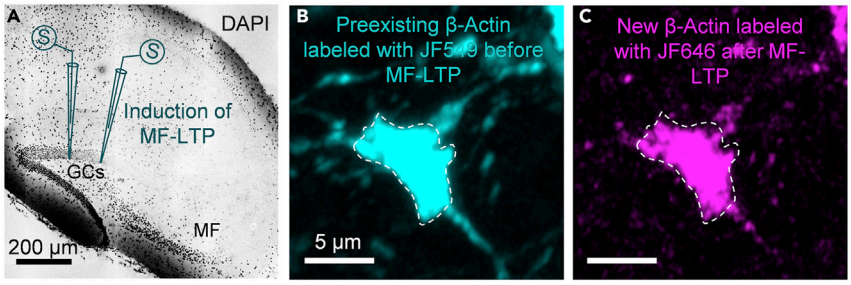

Figure 3. Expected outcome of LTP experiment

(A) Representative fluorescence image of DAPI-stained hippocampal slice to show the cut severing GC somas from MF axons acquired at 10× magnification.

(B and C) Representative images of pulse-chase labeled MFB before (B) and after (C) LTP acquired at 63× magnification. Please refer to Monday and Kharod et al.[1] for entire experiment.

In this section, we describe how to use image analysis software FIJI to quantify and analyze the fluorescence intensity of HaloTag-labeled fluorescence signal. Use of Zeiss LSM 880 required no additional steps after Airyscan processing and importing into FIJI.

1. Open the experiment file (.czi for Zeiss microscopy) using FIJI software (Figure 4A).

   Note: Usage of other microscopes may result in experimental file with other file formats.

2. Step 1 will open "Bio-formats import options" window. Select "Hyperstack" in the "View Stack with:" under "Stack viewing" and press "OK" (Figure 4B).
3. Default setting should lead to two separate channels as indicated by the sliding bar at the bottom of the window. Adjust brightness, contrast, maximum and minimum to evaluate images in both channels and to make sure pixel intensities are in a dynamic range and not saturated (Figures 4C and 4D). Per bouton, determine where in the z-stack the bouton begins and ends.
4. Under "Image" click "Stacks" and then select "Z project…" (Figure 4E).
5. Max z-project entirety of bouton (Figure 4F).
6. New image window will appear (Figure 4G).
7. In FIJI selection bar that says "(FIJI Is Just) ImageJ" select freestyle/hand drawing ROI selection tool. Since Halo-actin fills the entirety of the bouton, draw ROI in "pre-existing actin" or "JF549" channel, and measure mean pixel intensity (Analyze → Measure) (Figure 4H). MFBs are identified based on both morphology and the cell-specific expression of Halo-actin in GCs. While MF axons have both giant MFBs and small en passant bouton synapses onto local interneurons, the two are easily distinguishable by the large size and complexity of the former, which are 5–50 μm^3 in volume (when measured by confocal microscopy) and often have multiple filopodial protrusions. Due to the anatomy of the hippocampus, targeting the presynaptic terminal results in only MFB fluorescent labeling in the area being imaged (CA3).
8. Leave consistent for "newly synthesized actin" or "JF646" channel. It is important to keep ROI consistent in both channels. Measure mean intensity (Figure 4I). You can now export these measurements into Prism or equivalent software.

LIMITATIONS

This protocol outlines a novel use of the HaloTag self-labeling system. It is versatile like GFP and is bright enough to provide a direct measurement of specific proteins of interest in tissue without the need for amplification. It has pushed the bounds of protein imaging capabilities; traditional protein tagging systems have been limited to biochemical isolation and purification. The HaloTag system has overcome this limitation and has been used in a wide array of applications.[14] There are a few limitations of this method, however, that may prove cumbersome for widespread adoption. First,

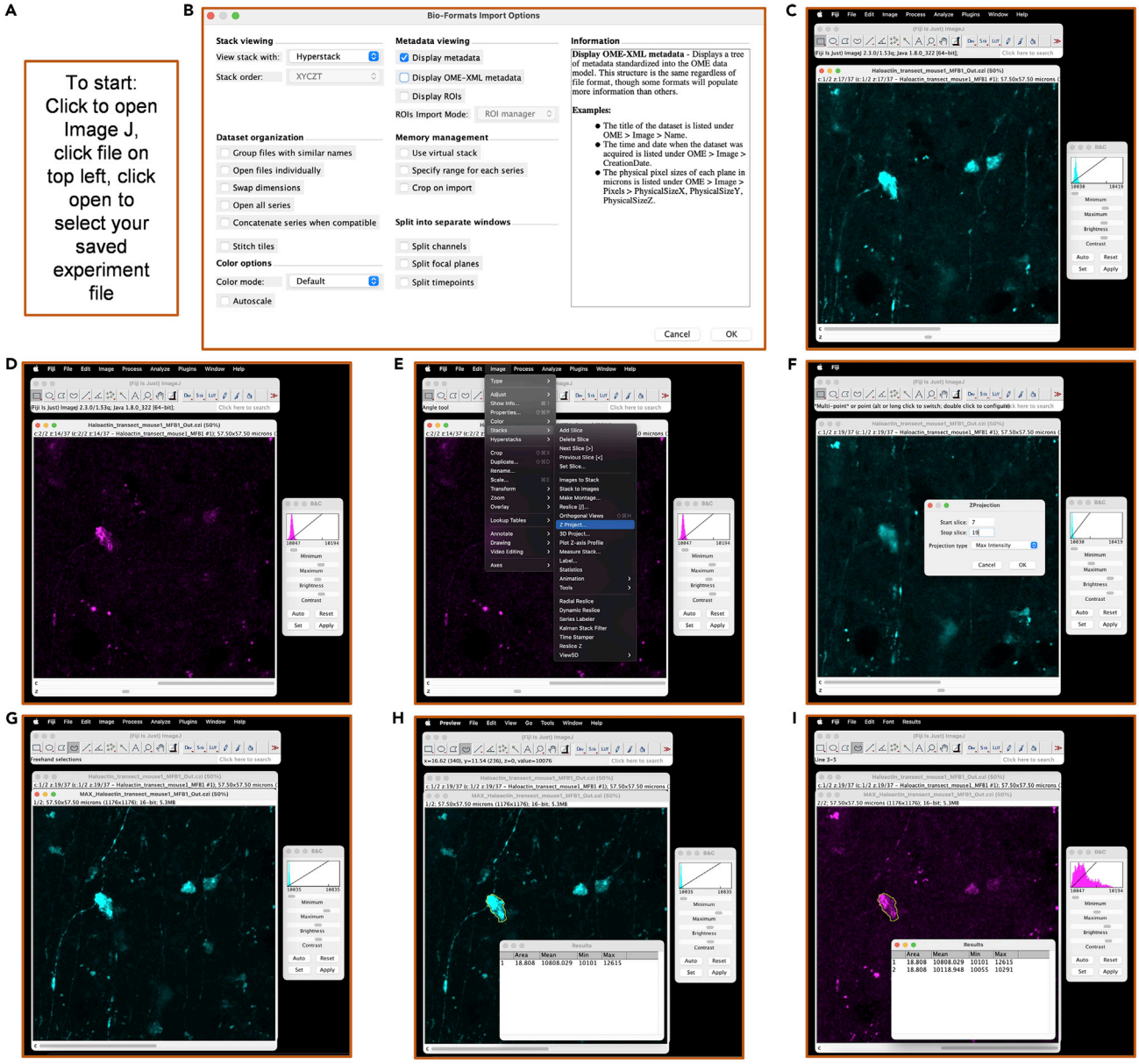

Figure 4. Analysis using FIJI software

(A–I) Screenshots illustrating step-by-step how to use FIJI software to analyze fluorescence images of MFBs after experimental manipulations.

researchers have to design their own HaloTag reporter construct if one does not currently exist for their application of interest.[14] Extensive control experiments should be conducted prior to pulse-chase labeling to make sure addition of tag did not result in an inactive protein or changes in protein function and may require attaching the HaloTag protein to the other terminus or other part of the protein.

Use of acute tissue is associated with some limitations. If slices are not properly oxygenated during the protocol there could be less protein synthesis, as local translation and plasticity are both energy-dependent processes.[15,16] Depending on the tissue type and different types of cell cultures, concentrations of dyes should be adjusted to make sure they are at saturating levels and wash steps may need to be optimized to reduce background. Washes with ACSF during the pulse chase assay are

critical for decreasing background as much as possible. For our tissue imaging, HaloTag labeling could not presently detect rapid changes (seconds range events) as time needed for labeling and washes occurred on the minutes timescale. In regard to presynaptic imaging, if your synapse of interest has extremely small presynaptic terminals, it may be difficult to adequately measure differences in signal over background. Incorporating biosensors such as pH-based measurements (synapto-pHlourins), other bright fluorescent tags that allow bulk measurements may be useful and better suited as success of this protocol is dependent on maximizing signal-to-noise ratio, if your protein of interest allows. Lastly, exposure of slices to light should be minimized to reduce photobleaching.

While this protocol has focused on the HaloTag, there are other commercially available alternative protein tagging systems that could also be more suitable for your purposes and experimental goals.[17–22] While all fusion tag-based systems are designed to label a protein of interest, the main difference between systems usually depends on the type of tag utilized (peptide vs chemical) and the mechanism. Depending on your purposes and downstream experiments, orthogonal-tagging systems can be used together but cross-reactivity may need to be considered.

TROUBLESHOOTING

Problem 1

From step 8. Missed injection site.

Potential solution

The most common problem when targeting small subcortical structures such as hippocampal subdivisions is to have fluorescently labeled neurons outside the intended target region. It may arise from incorrect or missed injection coordinates or inadequate positioning of the mouse skull in the stereotaxic frame during injection.

It is vital to ensure that the skull is level during the injection of viral constructs. This can be double-checked by measuring bregma and lambda height with the needlepoint. Also, the head should not be tilted to either side and this can often be checked by eye. The skull fissure should appear straight as an indication of level positioning side-to-side, when visualized from the top.

When performing the craniotomy, it is important to drill through the entirety of the bone. Any residual bone can deflect the Hamilton tip and infuse virus into wrong structure. To avoid drilling too far down and injuring the brain, one can drill and then pick at skull with a needle tip to ensure the hard bone structure has been drilled through.

When the needle is being lowered into the region of interest, you can slowly insert the needle 0.1 units below targeted region and then pull back up before infusing virus to create a reservoir for the volume that is about to enter. New users may prefer to practice with a methylene blue solution instead of virus until they are comfortable hitting the target region.

Additionally, you can use antibodies against HaloTag (1:500) or your gene of interest to confirm the location of the injection site if need be.

Problem 2

From step 18. Synaptic stimulation or other manipulations do not result in changes in protein synthesis.

Potential solution

It is possible that an insufficient recovery after slicing, inadequate stimulation parameters or wrong positioning of the stimulation pipette can result in undetectable changes in protein synthesis where expected. Hypoxic brain damage may occur if brain dissection takes longer than indicated. Slicing and recovery solutions should have correct pH, osmolarity and temperature.

As indicated above, brain slices need continuous saturation with carbogen gas. While some interruptions may be required to pick up and move slices, it is important to keep all such interruptions to a minimum. It is also important to bubble solutions for a minimum of 5–10 min before adding slices. If you are using animals that are older than 2–3 months, it may be helpful to perform an intracardial perfusion with ice-cold ACSF.

For synaptic stimulations, it may be important to adjust your stimulation protocol depending on your synapse and cell type of interest. For example, a protocol that induces long term changes in plasticity at one synapse may lead to increases in protein synthesis at that synapse but may lead to no changes in another synapse. It is, therefore, important to adjust all manipulations to your particular region of interest.

Problem 3

From step 18. Protein synthesis inhibitors do not block signal of second dye.

Potential solution

Translation inhibitors block different steps of protein synthesis. It may be necessary to test different concentrations and durations to achieve complete inhibition. Depending on your protein of interest, additional controls for your application may have to be conducted. Alternatively, dye concentrations may not be saturating and will have to be optimized empirically.

Problem 4

From step 21. Brain slices move on the glass slide during mounting.

Potential solution

After gently transferring brain slices into the slide, dry the surrounding solution with a folded Kimwipe to avoid sections moving from the slides. Also, take caution to apply only necessary amount of mounting medium.

Problem 5

From steps 24–26. Fluorescence photobleaching.

Potential solution

The laser should be switched off by switching off the light source when image acquisition is complete. To avoid any light exposure in between sample preparation, storage and image acquisition, store all slides in a dark slide box. Be sure to protect samples from light. This is critical as measurements to make conclusions between treatment groups is driven by fluorescence intensity.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Pablo E. Castillo (pablo.castillo@einsteinmed.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data reported in this paper will be shared by the lead contact upon reasonable request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this work paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.C.K., P.E.C., H.R.M., Y.J.Y; Methodology, S.C.K., H.R.M., Y.J.Y.; Investigation, H.R.M., S.C.K.; Resources, Y.J.Y., P.E.C.; Writing – original draft, S.C.K., P.E.C.; Writing – review & editing, all authors; Visualization, H.R.M., S.C.K.; Supervision, H.R.M., Y.J.Y., P.E.C.; Funding acquisition, H.R.M., Y.J.Y., P.E.C.; Project administration, P.E.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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