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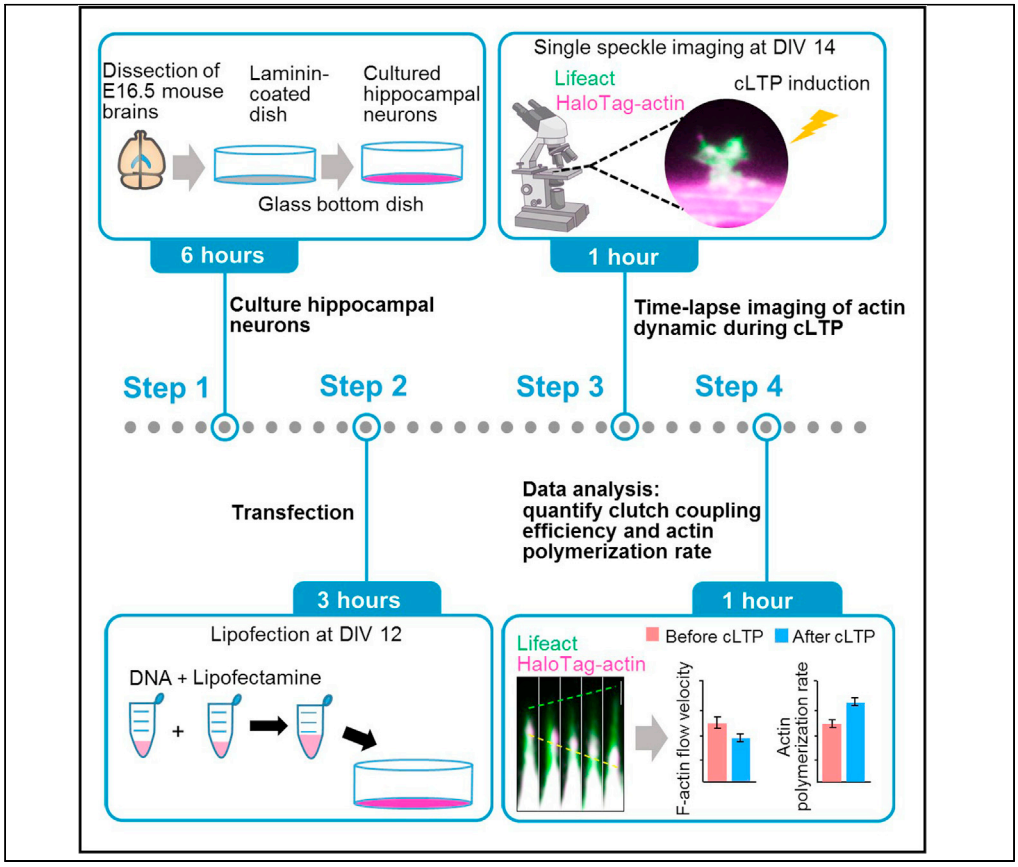
Simultaneous analyses of clutch coupling and actin polymerization in dendritic spines of rodent hippocampal neurons during chemical LTP



Dendritic spine enlargement by synaptic activation is thought to increase synaptic efficacy underlying learning and memory. This process requires forces generated by actin polymerization and actin-adhesion coupling (clutch coupling). Here, we describe a protocol to monitor actin filament retrograde flow and actin polymerization within spines using a standard epi-fluorescence microscope. In combination with chemical long-term potentiation, this protocol allows us to quantify clutch coupling efficiency and actin polymerization rate, which are essential variables for generating forces for activity-dependent spine enlargement.

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Highlights

Analysis of F-actin retrograde flow using a standard epi-fluorescence microscope

F-actin flow velocity reflects the efficiency of clutch coupling

Analysis of actin polymerization rate using a standard epi-fluorescence microscope

Analysis of these variables in dendritic spines during chemical LTP induction

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Simultaneous analyses of clutch coupling and actin polymerization in dendritic spines of rodent hippocampal neurons during chemical LTP

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SUMMARY

Dendritic spine enlargement by synaptic activation is thought to increase synaptic efficacy underlying learning and memory. This process requires forces generated by actin polymerization and actin-adhesion coupling (clutch coupling). Here, we describe a protocol to monitor actin filament retrograde flow and actin polymerization within spines using a standard epi-fluorescence microscope. In combination with chemical long-term potentiation, this protocol allows us to quantify clutch coupling efficiency and actin polymerization rate, which are essential variables for generating forces for activity-dependent spine enlargement.
For complete details on the use and execution of this protocol, please refer to Kastian et al. (2021).

BEFORE YOU BEGIN

Preparation of poly-D-lysine (PDL) and laminin-coated glass bottom dishes

Timing: 1 h

1. Coat glass bottom dishes (diameter 14 mm) with PDL 100 μg/mL in phosphate-buffered saline (PBS) for overnight (16–24 h).
2. Wash out dishes 3 times with PBS.
3. Coat the dishes sequentially with laminin 10 μg/mL in PBS for overnight (16–24 h).

Preparation of primary cultured hippocampal neurons

Timing: 6 h

All relevant aspects of the experimental procedures were approved by the Institutional Animal Care and Use Committee of Nara Institute of Science and Technology and Kyoto University Graduate School of Medicine.

Note: refer to "materials and equipment" for the required solutions/media.

4. Place embryonic day 18 (E18) rats or E16 mice on ice.
5. Dissect out the brains from the skull and place in a sterile 10 cm dish containing 10 mL of ice-cold solution G.

Note: Perform brain dissection in a laminar air flow hood.

6. Under a stereo microscope, carefully remove the meninges of the cerebral hemispheres and dissect out the hippocampi.
7. Transfer the hippocampi into ice-cold solution B in a 15 mL centrifuge tube.
8. Incubate the hippocampi at 37°C with a water bath for 20 min.
9. Remove the solution B and add 3 mL of solution A.
10. Mechanically dissociate the hippocampi by gently pipetting with a Pasteur pipette (4 times).
11. Incubate hippocampi at 37°C with a water bath for 20 min.
12. Collect the supernatant containing dissociated neurons into a 50 mL centrifuge tube and add 3 mL of new solution A to the undissociated tissues in the 15 mL tube.
13. Repeat the steps 9–12 until the hippocampi are completely dissociated.

Note: Solution B contains papain, while solution A is without papain. These gradual digestion steps with papain causes less damage to neurons compared to a standard protocol with trypsin.

14. Remove white floating aggregates of DNA from the collected cell suspension by swirling and aspirating with a Pasteur pipette.
15. Centrifuge the cell suspension at 180 *g* at 4°C for 20 min and remove the supernatant.

Note: DNA is derived from damaged cells. DNA remained in the cell suspension will disturb the centrifugation. If you find neurons in the supernatant, centrifuge it again after carefully removing the DNA.

16. Add neurobasal medium (NB) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin to the cell pellet.
17. Count the cell number and seed hippocampal neurons on laminin-coated glass bottom dishes at the density 5 × 10^4 cells/dish with the final volume 500 μL of the medium.

⚠ CRITICAL: Complete from the brain dissection and to cell seeding within 2.5 h.

18. Incubate neurons at 37°C with 5% CO_2 in a CO_2 incubator for 3 h.
19. Replace the medium with NB medium containing 1X B27 and 1X GlutaMAX (NB maintenance medium).

Maintenance of cultured hippocampal neurons

Timing: 10–20 min

20. On days *in vitro* (DIV) 7, add 200 μL of new NB maintenance medium to the neurons in glass bottom dishes.

Note: Pre-warm the NB maintenance medium before adding to the neurons.

Transfection of plasmids encoding HaloTag-actin and Lifeact into neurons

Timing: 3 h

21. On DIV 12, transfer the conditioned medium from cultured neurons in glass bottom dishes to 15 mL centrifuge tube and add 500 μL of new NB maintenance medium to the neurons.

Note: Pre-warm and equilibrate NB maintenance medium at 37°C with 5% CO_2. Keep the conditioned medium at 37°C with 5% CO_2.

22. Incubate the cultured neurons at 37°C with 5% CO_2 for 30 min.
23. In a microfuge tube, dilute 3 μL of lipofectamine 2000 with 50 μL of NB maintenance medium, and then incubate it at room temperature (20°C–25°C) for 5 min.
24. In another microfuge tube, dilute 0.5 μg pFN21A-HaloTag-actin and 1 μg pmNeonGreen-N1-Lifeact into 50 μL of NB maintenance medium, and incubate it at room temperature (20°C–25°C) for 5 min.
25. Mix lipofectamine solution with DNA solution, and incubate the mixture at 37°C with 5% CO_2 for 20 min.

Note: The mixture contains total DNA and lipofectamine 2000 at a ratio of 1 (μg) : 2 (μL). This ratio is important for formation of the DNA-lipofectamine 2000 complex for transfection. The transfection efficiency in neurons is about 1.6%–2%.

26. Add 50 μL of the DNA-lipofectamine mixture to the neurons.
27. Incubate at 37°C with 5% CO_2 for 2.5 h.
28. Replace the DNA-lipofectamine medium with the conditioned medium collected at step 21.
29. Maintain the cultured neurons at 37°C with 5% CO_2 until DIV 14.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Poly-D-lysine hydrobromide	Sigma	Cat# P6407-5MG
Laminin solution from mouse EHS tumor	Wako	Cat# 120-05751
HaloTag TMR ligand	Promega	Cat# G8251
Tetrodotoxin	Wako	Cat# 206-11071
Bicuculin	Tocris	Cat# 0130
Strychnine	Nacalai Tesque	Cat# 32316-74
Glycine	Nacalai Tesque	Cat# 09591-55
Papain	Nacalai Tesque	Cat# 26036-34
DNaseI	Sigma	Cat# DN25-100MG
BSA	Sigma	Cat# A7906-10G
D-glucose	Nacalai Tesque	Cat# 16806-25
B-27 supplement 50X	Thermo Fisher Scientific	Cat# 17504-044
Neurobasal Medium	Thermo Fisher Scientific	Cat# 21103-049
Penicillin-Streptomycin	Nacalai Tesque	Cat# 26253-84
GlutaMAX ™-I (100X) Supplement	Thermo Fisher Scientific	Cat# 35050-061
PBS pH 7.4 (10X)	Thermo Fisher Scientific	Cat# 70011-044
Fetal Bovine Serum	Thermo Fisher Scientific	Cat# 10270-106
NaCl	Nacalai Tesque	Cat# 31320-05
KCl	Nacalai Tesque	Cat# 28514-75
$CaCl_2$ anhydrous	Wako	Cat# 039-00475
$MgCl_2$ Hexahydrate	Nacalai Tesque	Cat# 20909-55
HEPES	Nacalai Tesque	Cat# 17546-34
Critical commercial assays		
Lipofectamine 2000	Thermo Fisher Scientific	Cat# 11668027
Experimental models: Organisms/strains		
Mouse C57BL/6	Japan SLC; CLEA Japan	N/A
Rat Wistar	Japan SLC; CLEA Japan	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
pFN21A-HaloTag-actin	(Minegishi et al., 2018)	N/A
pmNeonGreen-N1-Lifeact	(Kastian et al., 2021)	N/A
Software and algorithms		
ImageJ software	National Institutes of Health	https://imagej.nih.gov/ij/index.html; RRID: SCR_003070
Microsoft Excel	Microsoft	https://www.microsoft.com/
GraphPad Prism7	GraphPad Software	https://www.graphpad.com/; RRID: SCR_002798
ZEN software	Carl Zeiss	https://www.zeiss.com/microscopy/int/products/microscope-software/zen.html; RRID: SCR_013672
Other		
Glass bottom dish	Matsunami	Cat# D1130H
Zeiss AxioObserver Z1	Zeiss	Cat# 431007-9902-000
CMOS camera	Hamamatsu Photonics ORCA-Flash4.0 V2	Cat# C11440-22CU

MATERIALS AND EQUIPMENT

- Solutions for primary cultured hippocampal neurons

Reagent	Final concentration	Amount
Solution G		
D-Glucose	22.2 mM	4 g
10× PBS	1× PBS	100 mL
MilliQ water	n/a	Up to 1L
Total	n/a	1 L

Note: Sterilize with vacuum filter and dispense the 20 mL of solution G to 50 mL tubes and store at −30°C for up to 6 months.

Reagent	Final concentration	Amount
Solution A		
BSA	15 μM	1 g
DNaseI	0.19 μM	6 mg
D-Glucose	10 mM	1.8 g
10× PBS	1× PBS	100 mL
MilliQ water	n/a	Up to 1L
Total	n/a	1 L

Note: Sterilize with vacuum filter and dispense the 10 mL of solution A to 15 mL tubes and store at −30°C for up to one month.

Reagent	Final concentration	Amount
Solution B		
Solution A	n/a	200 mL
Papain	0.5 mg/mL	100 mg
Total	n/a	200 mL

Note: Sterilize with vacuum filter and dispense the 4 mL of solution B to 15 mL conical tubes and store at −30°C for up to one month.

- Media for culturing long-term hippocampal neurons

Reagent	Final concentration	Amount
Neurobasal (NB) medium with FBS		
Neurobasal medium	n/a	44.5 mL
Fetal Bovine Serum (FBS)	10%	5 mL
Penicillin-Streptomycin	1%	500 µL
Total	n/a	50 mL

Note: Store at 4°C for up to 2 weeks.

Reagent	Final concentration	Amount
Neurobasal (NB) maintenance medium		
Neurobasal medium	n/a	48.5 mL
B27 supplement	1X	1 mL
GlutaMAX	1X	500 µL
Total	n/a	50 mL

Note: Store at 4°C for up to 2 weeks.

- Artificial cerebrospinal fluids (ACSFs)

Reagent	Final concentration	Amount
Stock ACSF solution containing $MgCl_2$		
NaCl	143 mM	4.18 g
KCl	5 mM	186 mg
$CaCl_2$ anhydrous	2 mM	111 mg
$MgCl_2$ Hexahydrate	1 mM	102 mg
D-Glucose	30 mM	2.7 g
HEPES	10 mM	1.19 g
MilliQ water	n/a	Up to 500 mL

Note: Adjust pH to 7.4 and sterilize with vacuum filter. Store the stock solution at 4°C for up to one month.

Reagent	Final concentration	Amount
Stock ACSF solution without $MgCl_2$		
NaCl	143 mM	4.18 g
KCl	5 mM	186 mg
$CaCl_2$ anhydrous	2 mM	111 mg
D-Glucose	30 mM	2.7 g
HEPES	10 mM	1.19 g
MilliQ water	n/a	Up to 500 mL

Note: Adjust pH to 7.4 and sterilize with vacuum filter. Store the stock solution at 4°C for up to one month.

Reagent	Final concentration	Amount
Glycine stimulation solution		
ACSF solution without $MgCl_2$	n/a	10 mL
1 mM Tetrodotoxin	0.5 μM	5 μL
3 mM Strychnine	1 μM	3.3 μL
20 mM Bicuculine	20 μM	10 μL
0.2 M Glycine	200 μM	10 μL
Total	n/a	10 mL

Note: Freshly prepare and store at 4°C for not more than one day.

STEP-BY-STEP METHOD DETAILS

TMR ligand treatment (on DIV14)

Timing: 2 h

The pFN21-HaloTag-actin protein covalently binds with fluorescent tetramethylrhodamine (TMR) ligand. The fluorescent TMR ligand allows to monitor the retrograde flow of HaloTag-actin speckles within dendritic spines.

1. Dilute 1 μL of TMR ligand in 200 μL of PBS as a working stock.

   Note: Diluted TMR ligand can be stored in the dark at 4°C for up to one week.

2. Collect 180 μL of the conditioned medium from cultured neurons and mix it with 20 μL of the TMR ligand working stock.
3. Replace the remaining conditioned medium of cultured neurons with the 200 μL TMR ligand-medium mixture.
4. Incubate at 37°C with 5% CO_2 for 1 h.
5. Wash the TMR ligand 3 times with pre-warmed PBS.
6. Incubate neurons with 500 μL of pre-warmed ACSF containing $MgCl_2$, 0.5 μM tetrodotoxin (sodium channel blocker), 1 μM strychnine (glycine receptor antagonist), 20 μM bicuculline (GABAa receptor antagonist) at 37°C with 5% CO_2 for 1 h.

Time-lapse imaging of dendritic spines before and after chemical long-term potentiation (cLTP)

Timing: 1 h

We use an epi-fluorescence microscope Axio Observer Z1 (Zeiss) equipped with a complementary metal oxide semiconductor (CMOS, ORCA Flash 4.0 V2, Hamamatsu) camera and a Plan-Apochromat 100x immersion oil lens with numerical aperture 1.40 (Zeiss). We also use ZEN imaging software to acquire images. Other Epi-fluorescence microscopes and imaging software with equivalent performances can be also used in this analysis.

Alternatives: CMOS camera and a plan-Achromat 100x lens are preferable components in standard epi-fluorescence microscope to monitor a retrograde flow of actin speckles in dendritic spines. However, epi-fluorescence microscopes with equivalent components are also applicable.

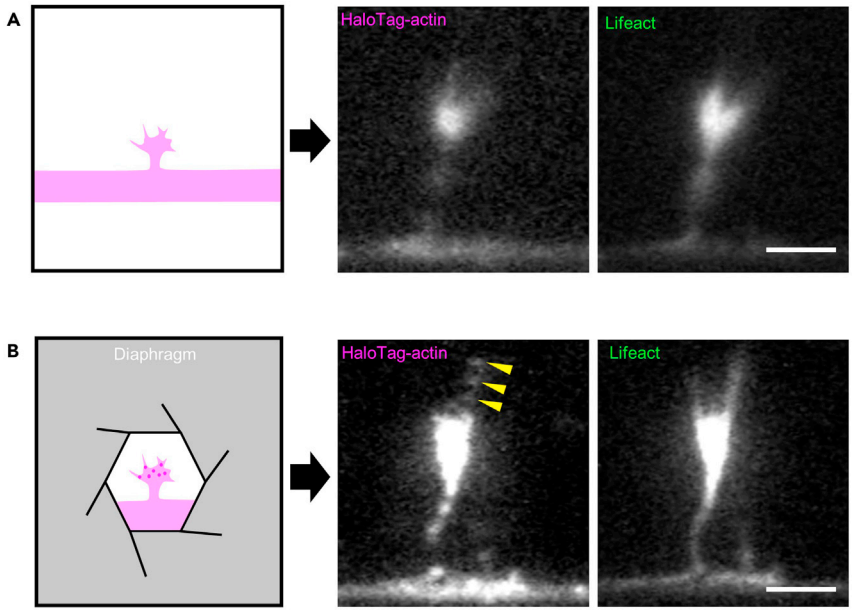

Figure 1. Representative fluorescence images of HaloTag-actin under a full-opened and reduced field of diaphragm
(A) HaloTag-actin signals are dim in a fully illuminated field.
(B) When the field of the microscope diaphragm is reduced to illuminate a minimum area including the spine, clear HaloTag-actin speckles (yellow arrowheads) will be seen in the spine (right). Scale bars: 1 μm for (A) and (B).

7. Set the stage-top incubator of the microscope at 37°C.

   Note: Epi-fluorescence microscope is equipped with stage-top incubator without CO_2 regulation system. During live imaging, cultured hippocampal neurons are maintained in ACSF solution containing 10 mM HEPES which enables the pH maintenance without CO_2. Thus, the neuronal conditions are not affected substantially in the absence of 5% CO_2.

8. Transfer the TMR ligand-treated neurons in the incubator.
9. Select neurons that are strongly expressing Lifeact and weakly expressing HaloTag-actin.

   Note: Lifeact visualizes F-actins in dendritic spines, while HaloTag-actin allows to monitor the speckles of actin molecules along F-actins. A high level of Lifeact expression ensures visualization of spine morphology; a low level of HaloTag-actin expression is essential for tracing single actin molecules.

10. Adjust the optimum exposure times of the two filter channels and close the field of the diaphragm to illuminate a minimum area including a dendritic spine (Figure 1).

    ⚠ CRITICAL: Illumination of a minimum area is a key for successful speckle imaging (Figures 1 and 2, and Methods video S1). To increase the S/N ratio, we recommend to illuminate the target spine without reducing the light by neutral density (ND) filters.

11. Acquire single plane time-lapse images of a spine for both HaloTag-actin and Lifeact every 2 s for 100 s.
12. To induce cLTP, replace ACSF solution containing $MgCl_2$ to freshly prepared glycine stimulation solution and incubate for 4 min.

    ⚠ CRITICAL: To avoid a shift of the spine location, replace the solution without touching the dish.

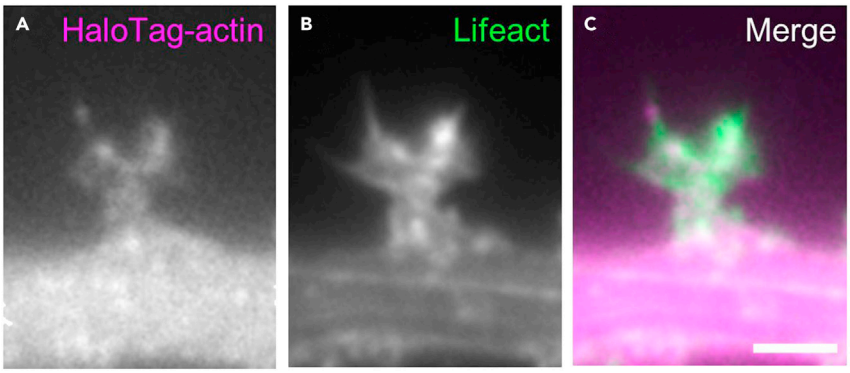

Figure 2. Dual-color fluorescence images of F-actin and HaloTag-actin in mushroom spine
(A–C) Representative images of HaloTag-actin (A), Lifeact (B) and an overlay (C) in a mushroom spine. Scale bar: 2 μm. See also Methods video S1.

Note: To minimize a focus drift due to thermal changes of the microscope, pre-warm glycine stimulation solution and ACSF without $MgCl_2$ before replacing.

13. Replace glycine stimulation solution with ACSF without $MgCl_2$ and immediately acquire time-lapse images every 2 s for 100 s.

EXPECTED OUTCOMES

Dendritic spine enlargement by synaptic activation is thought to increase synaptic efficacy underlying learning and memory (Honkura et al., 2008; Bosch et al., 2014). F-actin retrograde flow in spines has been monitored by using photoactivatable fluorescent proteins or by combination of super resolution microscope and fluorescent proteins (Honkura et al., 2008; Tatavarty et al., 2009, 2012; Frost et al., 2010; Chazeau et al., 2014). However, photoactivation of fluorescent proteins requires irradiation light with specific wavelength and intensity, which induces photobleaching and causes phototoxicity to cells (Lippincott-Schwartz and Patterson, 2009; Banaz et al., 2019).

Here we describe a protocol to monitor F-actin retrograde flow using a standard epi-fluorescence microscope without photoactivation; we can observe clear F-actin retrograde flow in spines by a simple procedure (Figure 2 and Methods video S1). A previous study reported that tracing actin speckles in flowing F-actins enable us to measure F-actin flow velocity (Figure 3) (Watanabe and Mitchison, 2002), which reflects the efficiency of clutch coupling. The clutch coupling transmits the force of F-actin retrograde flow to the substrate as traction force; concurrently, it reduces the F-actin flow velocity, thereby converting actin polymerization into the force that protrudes the leading-edge membrane (Mitchison and Kirschner, 1988; Suter and Forscher, 2000; Toriyama et al., 2013). By labeling F-actin with Lifeact, we can also visualize F-actin protrusion and quantify actin polymerization rate (Figure 4). Both the efficiency of clutch coupling and actin polymerization are essential variables for generating forces for spine enlargement (Kastian et al., 2021).

The induction of cLTP with glycine activates NMDA receptor and downstream signaling pathways for spine structural plasticity (Hruska et al., 2018). Thus, the analysis of F-actin retrograde flow and actin protrusion rate under cLTP can quantify the changes in the efficiency of actin-adhesion coupling (clutch coupling) and actin polymerization rate during activity-dependent spine enlargement (Figure 5). The present protocol is focused on the monitoring of the F-actin retrograde flow and actin polymerization rate; the method to analyze the changes of spine size during cLTP induction is described in our recent report (Kastian et al., 2021). In addition, this method can be also applied for the analyses of clutch coupling and actin polymerization in growth cones (Toriyama et al., 2013; Minegishi et al., 2018).

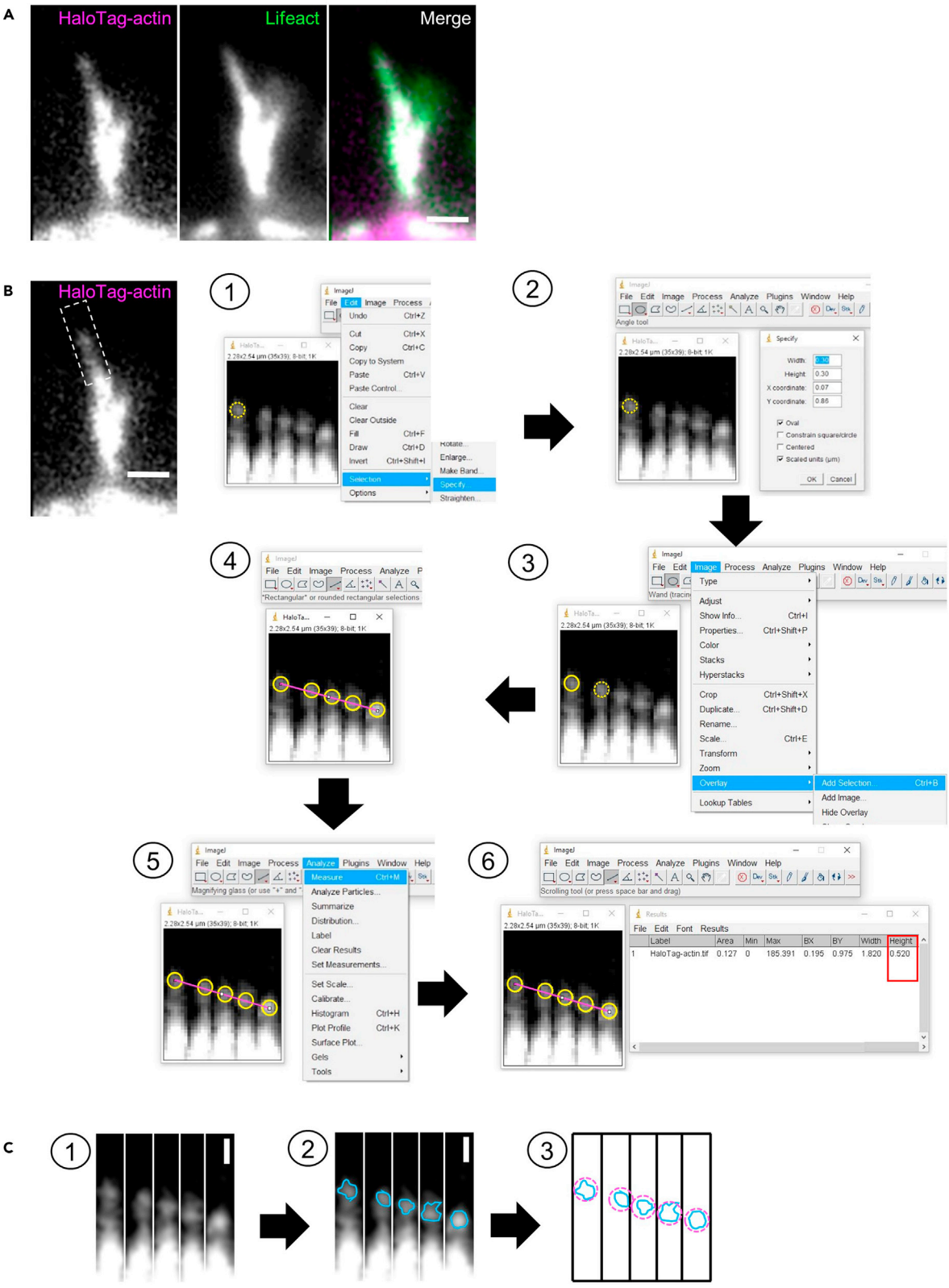
A
HaloTag-actin
Lifeact
Merge
B
HaloTag-actin
1
2
3
4
5
6
C
1
2
3

Figure 3. Analysis of F-actin retrograde flow

(A) Representative images of HaloTag-actin and Lifeact in a mushroom spine and their overlay.

(B) A time-lapse montage image of HaloTag-actin were analyzed from the indicated rectangle region (left) using ImageJ: ① Select 'Oval Selection' in the toolbar and draw a circle over the speckle, then click Edit -> Selection -> Specify; ② Specify the size of the circle to 300 nm width and 300 nm height; ③ Adjust the circle position and determine the center of the speckle. Then click Image->Overlay->Add Selection to overlay the circle on speckles; ④ Select 'Straight Line' in the toolbar and draw a line that links the centers of the circles (magenta line); ⑤ Click Analyze->Measure to calculate the translocation distance of the speckle; ⑥ Height indicates the translocation distance of the actin speckle in 10-s (red box).

(C) Overlay circles on irregular shape of actin speckles. ① A time-lapse montage of HaloTag-actin; ② Irregular shape and size of actin speckles as shown in blue lines; ③ Overlay circles on the speckles to determine the center of the speckles and to precisely track the retrograde movement of the speckles. Scale bar: 1 μm for (A) and (B, left); 0.5 μm for (C).

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of F-actin flow velocity and polymerization rate using ImageJ

As the signals of HaloTag-actin is weak and the distal tip of the spine head is very thin, we recommend to adjust the contrast and brightness of the signals for optimizing the analyses of F-actin flow velocity and protrusion rate.

> *Note:* Multi Gauge software (Fujifilm) is also applicable for the quantification F-actin flow velocity and polymerization rate. Alternatively, 'Manual Tracking' plugin of ImageJ also allows to track the retrograde flow of F-actin.

1. Run ImageJ and open the time-lapse images.
2. Select a region of a protrusion emanating from a spine head in which a clear retrograde movement of HaloTag-actin speckles (rectangle, Figure 3B, left) and Lifeact signals (rectangle, Figure 4A, left) are observed. Rotate the image so that the protrusion directs to upward.
3. Click Image->Duplicate to duplicate the selected region which shows actin flows retrogradely for at least five time-frames.
4. Click Image->Stack->Make Montage of the time-lapse images of HaloTag-actin (Figure 3B, ①) and Lifeact (Figure 4A, ①) for the selected region.
5. Quantify F-actin flow velocity and actin polymerization rate.
   a. Quantification of F-actin flow velocity (Figure 3B):
      i. Select 'Oval Selection' in the toolbar and then click Edit -> Selection -> Specify to draw 300-nm-scale of circle over the speckle (Figure 3B, ①-②).
      ii. Adjust the circle position and determine the center of the speckle. Then, click Image->Overlay->Add selection to overlay the circle on the speckle (Figure 3B, ③).

> ⚠ CRITICAL: As the size and shape of speckles is not regular (Figure 3C), overlaying 300-nm-diameter circle scales is necessary to determine the center of the speckles and to precisely track the retrograde movement of the speckles.

      iii. Select 'Straight Line' in the toolbar and draw a line that links the centers of the circles (magenta line, Figure 3B, ④).
      iv. Click Analyze->Measure to calculate the translocation distance of the speckle; Height indicates the translocation distance of the actin speckle in 10-s (Figure 3B, ⑤-⑥).
   b. Quantification of F-actin polymerization rate (Figure 4A):
      i. Increase the brightness of the image and select 'Straight Line' in the toolbar to draw a line that links the tips of the F-actin protrusion (magenta, Figure 4A, ①).
      ii. Measure the change in the length of the protrusion and calculate actin extension rate (Figure 4A, ②-③).
      iii. F-actin polymerization rate is calculated as sum of the F-actin flow velocity and extension rate (red double-headed arrow, Figure 4B).

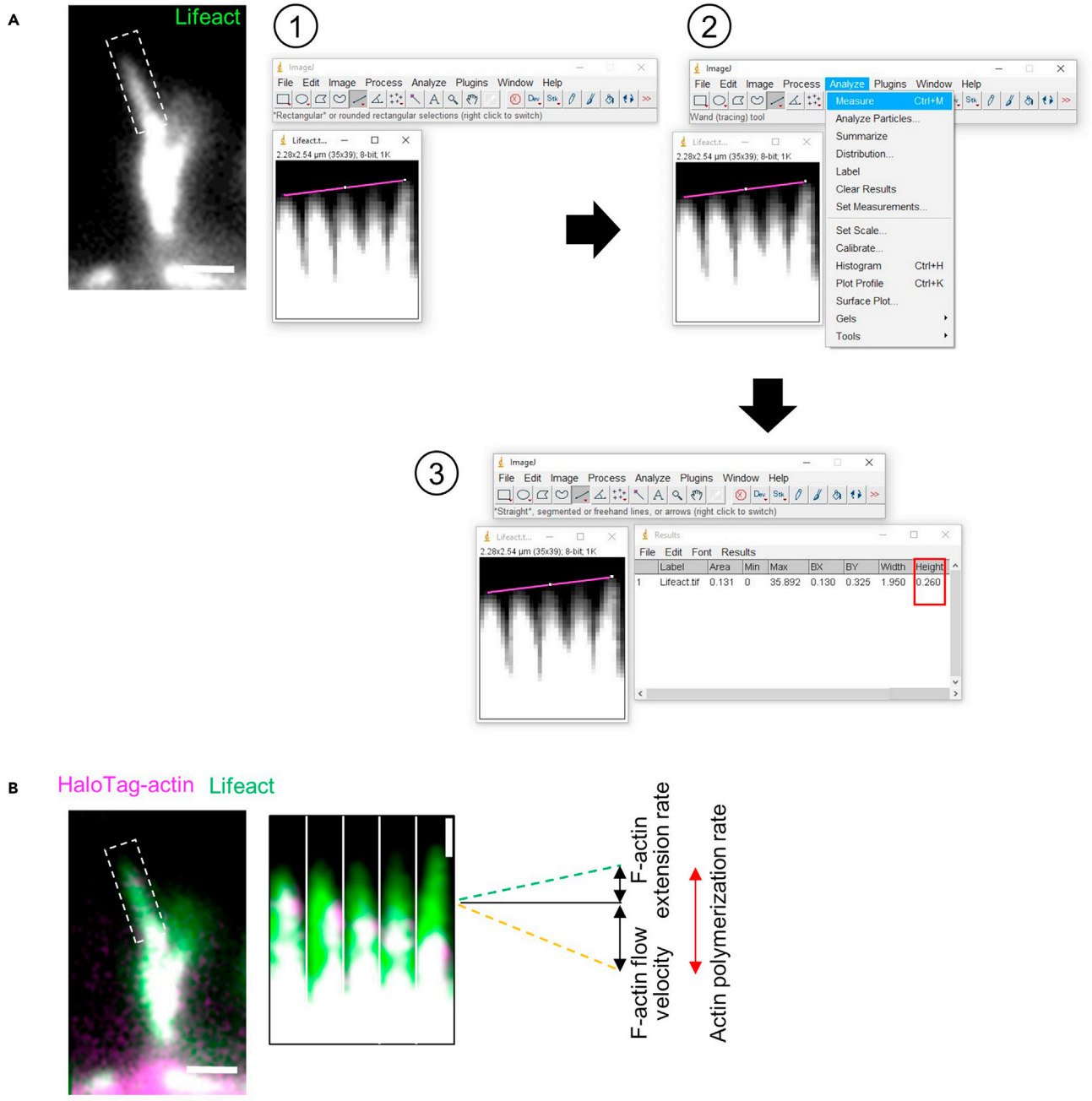

Figure 4. Analyses of actin polymerization rate

(A) A time-lapse montage image of HaloTag-actin was analyzed from the indicated rectangular region using (left) ImageJ: ① Increase the brightness of the image and select 'Straight Line' to draw a line (magenta) that links the tips of the F-actin protrusion; ② Click Analyze->Measure to calculate the actin extension rate; ③ Height indicates the actin extension rate in 10-s (red box).

(B) Actin polymerization rate is calculated from the sum of F-actin flow velocity and actin extension rate (red double-headed arrow). Scale bars: 1 μm for (A) and (B), left; 0.5 μm for (B), right.

LIMITATIONS

Single speckle imaging using HaloTag-actin and Lifeact is a useful technique to analyze the efficiency of clutch coupling and actin polymerization rate during activity-dependent spine enlargement. However, it has limitations. First, protrusions of the spine head move dynamically; this movement

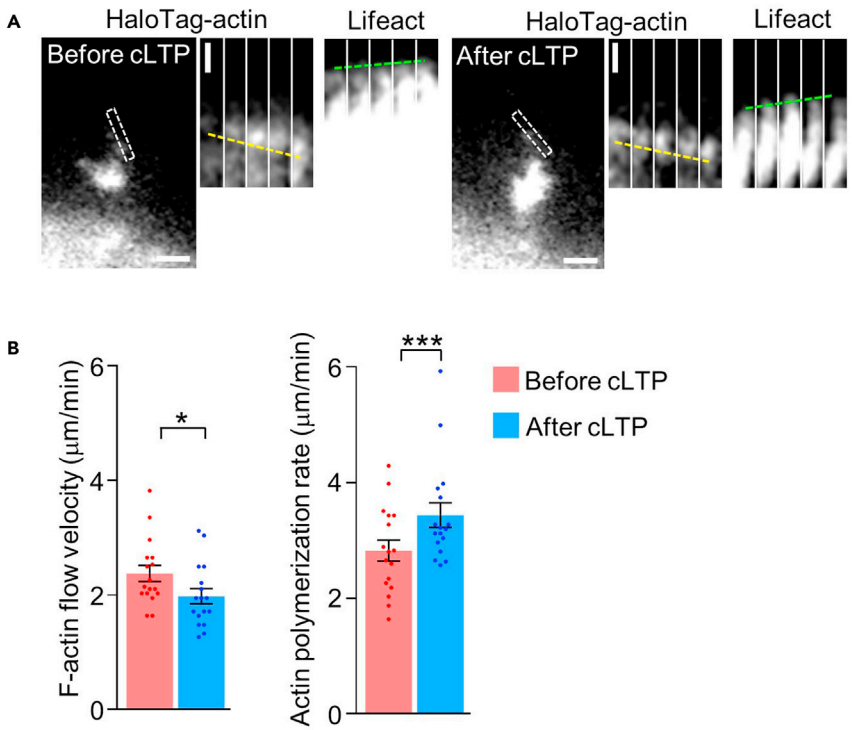

Figure 5. Representative data of the F-actin flow velocity and polymerization rate during cLTP

(A) Representative images of HaloTag-actin speckles (yellow dashed line) and Lifeact (green dashed lines) from indicated region (rectangular dashed lines) before (left) and after cLTP (right). Scale bars, 1 μm (left), 0.5 μm (right).

(B) F-actin flow velocity (left) and actin polymerization rate (right) calculated from fluorescent speckles images of HaloTag-actin and fluorescence images in dendritic spines. Data represent means ± SEM. ***$p < 0.01$; **$p < 0.02$; *$p < 0.05$; ns, not significant.

sometimes obstructs tracing the HaloTag-actin speckles for more than 10 s. Therefore, we recommend to select the protrusions which are relatively static for at least 60 s during time-lapse imaging. Second, induction of cLTP enlarges about 54% of spines, while 46% of spines are not responsive (Nägerl et al., 2008; Hruska et al., 2018; Kastian et al., 2021); thus, actin dynamics in some spines would not change after glycine treatment. Third, this protocol is not applicable for organotypic brain slices or acute slices, as the autofluorescence of thick slice cultures disturbs the tracing of actin speckles. Fourth, only one spine can be imaged in this protocol. The analysis of the effect of cLTP induction on clutch coupling efficiency and actin polymerization rate requires at least 16–22 spines per an experimental group.

TROUBLESHOOTING

Problem 1

Neurons die during long-term culture (before you begin: step 29).

Potential solution

There are two possible reasons. First, too low density of neurons decreases the survivability during long-term culture. Plating neurons at a density 40.000–50.000 cells per glass bottom dish (14 mm glass diameter) is optimum for 2-week culture. Second, lipofectamine is toxic for neurons. According to the manufacture protocol, the ratio of total DNA and lipofectamine 2000 should be 1 (μg) : 2 (μL). The optimum incubation time of the DNA-lipofectamine mixtures in cultured neurons is 2.5 h.

Problem 2

Spine location changes after cLTP induction (step-by-step method details: step 13).

Potential solution

Do not touch the dish when you replace the solution. In addition, focus drift occurs due to thermal changes of the microscope. We recommend to use pre-warmed glycine stimulation solution and ACSF without $MgCl_2$ to minimize the effect of thermal changes.

Problem 3

Signals of HaloTag-actin are too strong or too weak for tracing the retrograde movements of HaloTag-actin speckles (step-by-step method details: step 9).

Potential solution

Suitable plasmid concentration of pFN21A-HaloTag-actin for transfection is a critical point for single speckle imaging: 0.5 μg of pFN21A-HaloTag-actin is an optimum amount to observe clear retrograde movement of HaloTag-actin speckles. Actin fluorescently labeled with GFP or mRFP can be used for speckle imaging (Watanabe and Mitchison, 2002; Toriyama et al., 2013); however, signals of mRFP-actin speckles appears less clear in spines.

Problem 4

The signal of Lifeact is too weak at the protrusion tips of spine head (quantification and statistical analysis: step 5b i-iii).

Potential solution

The thickness of the distal tip of the protrusions emanating from the spine head is very small compared to the proximal spine head. Thus, we recommend to adjust the contrast and brightness of the montage image for analysis.

Problem 5

The spine head is not enlarged after cLTP induction (step-by-step method details: step 13)

Potential solution

As we discussed in the 'limitations' section, about 46% spines are non-responsive to cLTP induction (Hruska et al., 2018). Therefore, we recommend including the quantitative data of non-responsive spines in the statistical analysis.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Naoyuki Inagaki (ninagaki@bs.naist.jp).

Materials availability

No unique reagents were generated.

Data and code availability

No large data set or new software were developed

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100904.

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AUTHOR CONTRIBUTIONS

Designing of the protocols and writing, R.F.K., T.M., and N.I.; experimental data, T.M. and R.F.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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