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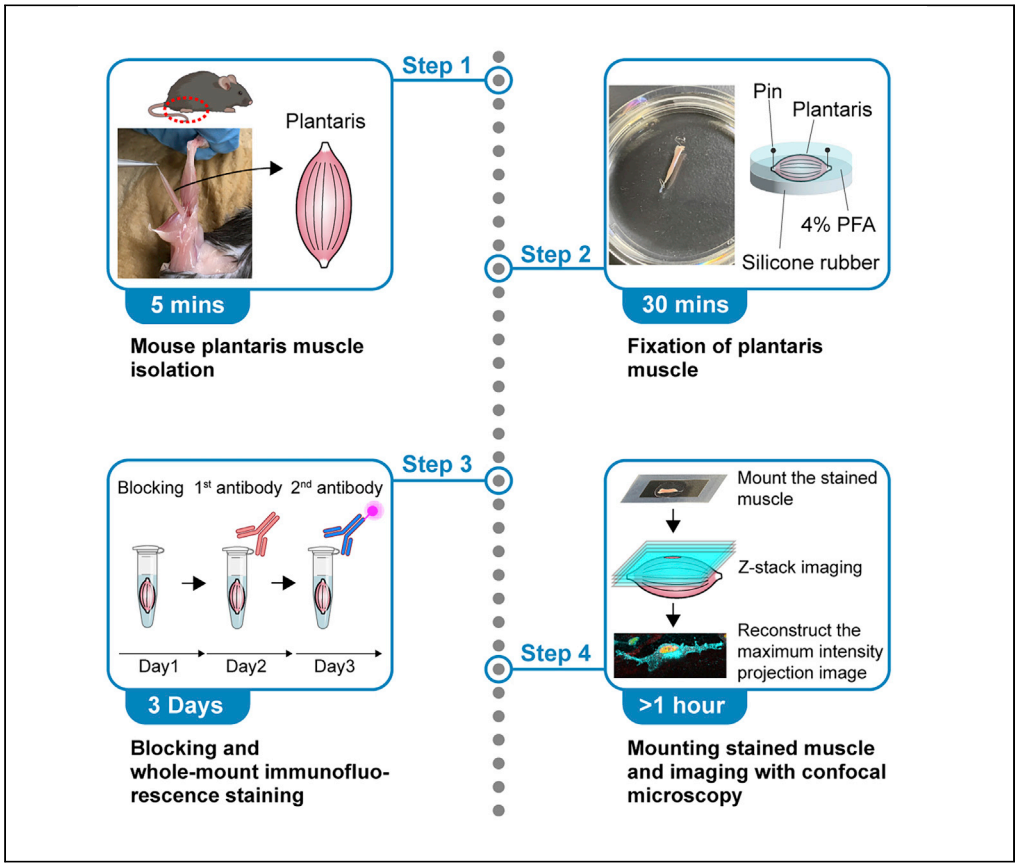
Whole-mount immunofluorescence staining of mesenchymal progenitors in murine plantaris muscle



We recently demonstrated that mesenchymal progenitors play a critical role in regulating satellite cell-dependent myonuclear accretion during overload-induced muscle hypertrophy. Here, we describe the detailed protocol for whole-mount immunofluorescence staining of mesenchymal progenitors in mouse plantaris muscle. Z-stack image reconstruction provides a whole-cell image and enables examination of YAP nuclear translocation in mesenchymal progenitors induced by overload.

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

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Highlights

Fixation of mouse plantaris muscle on a silicone rubber plate for whole-mount staining

Sandwiching stained muscle by cover glasses with silicone spacer for z-stack imaging

Whole-cell imaging of mesenchymal progenitors by z-stack image reconstruction

Examination of YAP nuclear translocation in mesenchymal progenitors induced by overload

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Whole-mount immunofluorescence staining of mesenchymal progenitors in murine plantaris muscle

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SUMMARY

We recently demonstrated that mesenchymal progenitors play a critical role in regulating satellite cell-dependent myonuclear accretion during overload-induced muscle hypertrophy. Here, we describe the detailed protocol for whole-mount immunofluorescence staining of mesenchymal progenitors in mouse plantaris muscle. Z-stack image reconstruction provides a whole-cell image and enables examination of YAP nuclear translocation in mesenchymal progenitors induced by overload.

For complete details on the use and execution of this protocol, please refer to Kaneshige et al. (2022a).

BEFORE YOU BEGIN

The protocol below describes the specific steps for the staining of murine plantaris muscles. However, we have also used this protocol for the staining of murine extensor digitorum longus and soleus muscles.

Institutional permissions

All animal experiments were approved by the Experimental Animal Care and Use Committee of Tokushima University, University of Tokyo and University of Osaka and were performed in accordance with the ARRIVE guidelines. Experimenters will need to acquire permissions from their relevant institutions.

Preparations for muscle fixation

Timing: 2 days

1. Make silicone rubber plate. See troubleshooting: problem 1.
   a. Mix 25 grams of liquid silicone rubber with 1.25 grams of curing agent with stirring by the magnetic stirrer for 5 min at 15°C–25°C (See the key resources table for material product information).

b. Immediately after mixing, pour the mixed solution to 60-mm or 100-mm dish and adjust the depth of the mixed solution to about 3 mm.
c. Allow the silicone rubber to cure for at least 1 day at 15°C–25°C.

2. Dissolve paraformaldehyde (PFA) powder in PBS.
   a. Weigh 5 g of PFA and add 50 mL of ultrapure water to create a 10% PFA solution.
   b. Add 100 μL of 10 N NaOH and warm the solution at 60°C–70°C.
   c. Mix by the magnetic stirrer for 30 min.
   d. Keep the solution on ice.
   e. To make 4% PFA, mix 10% PFA solution, 10× PBS, and ultrapure water at 4:1:5 immediately before use.

Note: Use freshly prepared 4% PFA solution for efficient fixation.

Note: It takes at least one day for the silicone to cure. Although it does not cure rapidly in a few minutes, it is recommended that the mixture be poured into the plate immediately after mixing.

Note: The silicone plate can be reused.

Note: To fully immerse the plantaris muscle, about 10 mL or 20 mL of PFA solution is required for the fixation in 60-mm or 100-mm dish, respectively.

Preparation of blocking solution for immunostaining

Timing: 10 min

3. Make blocking solution.
   a. Add 2 g of bovine serum albumin (BSA) and 0.5 mL of Triton X-100 to 49.5 mL of PBS to make blocking solution (4% BSA and 1% Triton X-100 in PBS). See troubleshooting: problems 3 and 4.
   b. Mix thoroughly.
   c. Keep the blocking solution at 4°C.
   d. For long term storage, store in aliquots at −20°C.

Note: After thawing, the blocking solution should be stored at 4°C and used within 2 weeks.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat polyclonal anti-mouse PDGFRα (2.5 μg/mL)	R&D	Cat#AF1062; RRID: AB_2236897
Rabbit monoclonal anti-YAP (clone D8H1X) XP(R) (1:100)	Cell Signaling Technology	Cat#14074S; RRID: AB_2650491
Alexa 647-conjugated donkey anti-goat IgG (H+L) (1:1000)	Jackson ImmunoResearch	Cat#705-605-147; RRID: AB_2340437
Alexa 594-conjugated donkey anti-rabbit IgG (H+L) (1:1000)	Jackson ImmunoResearch	Cat#711-585-152; RRID: AB_2340621
Chemicals, peptides, and recombinant proteins		
Liquid silicone rubber	Shin-Etsu Chemical	Cat#KE-103
Curing agent	Shin-Etsu Chemical	Cat#CAT-103
Paraformaldehyde	Wako	Cat#162-16065
Bovine serum albumin	Sigma-Aldrich	Cat#A7030
Triton X-100	Sigma-Aldrich	Cat#T8787
DAPI solution (1:5000)	Dojindo	Cat#D523
SlowFade Diamond Antifade Mountant	Thermo Fisher Scientific	Cat#S36972

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strdains		
Mouse: B6.129S4-*Pdgfra*$^{tm11(EGFP)Sor}$/J, male and female, from 3 to 5 months	The Jackson Laboratory	JAX: 007669; RRID: IMSR_JAX:007669
Software and algorithms		
LAS X software	Leica	https://www.leica-microsystems.com/
Other		
Forceps	FST	Cat#11231-20
Scissors	FST	Cat#14060-09
Intradermal needles, 0.14 × 5 mm	Seirin	NS type
Silicone rubber sheet, 0.5 mm thick	Wako	Cat#291-35641
NEO cover glass, thickness No. 1, 24 × 55 mm	Matsunami	Cat#C024551
TCS SP8 confocal laser scanning microscope	Leica	https://www.leica-microsystems.com/

STEP-BY-STEP METHOD DETAILS

Excision of mouse plantaris muscle

Timing: 5 min/mouse

To overload the plantaris muscle, tenotomy was performed according to the procedure described in our parallel STAR protocol (Kaneshige et al., 2022b), and the muscle was removed as described below.

1. Sacrifice the mouse (in our study, B6.129S4-*Pdgfra*$^{tm11(EGFP)Sor}$/J mice were used and hereafter referred to as 'PDGFRα^{EGFP}') and peel off the skin to expose the lower leg muscles.
2. Cut the Achilles tendon of the gastrocnemius and soleus muscles leaving the Achilles tendon of the plantaris muscle intact (Figures 1A and 1B).
3. Pinch the Achilles tendon of the gastrocnemius and soleus muscles by forceps and pull them away from the tibia to expose the plantaris muscle (Figure 1C).
4. Cut the Achilles tendon of the plantaris muscle.
5. Pinch the Achilles tendon of the plantaris muscle by forceps and pull them away from the tibia (Figure 1D).
6. Cut the proximal attachment site of the plantaris muscle to the femur and isolate the whole plantaris muscle (Figure 1E).

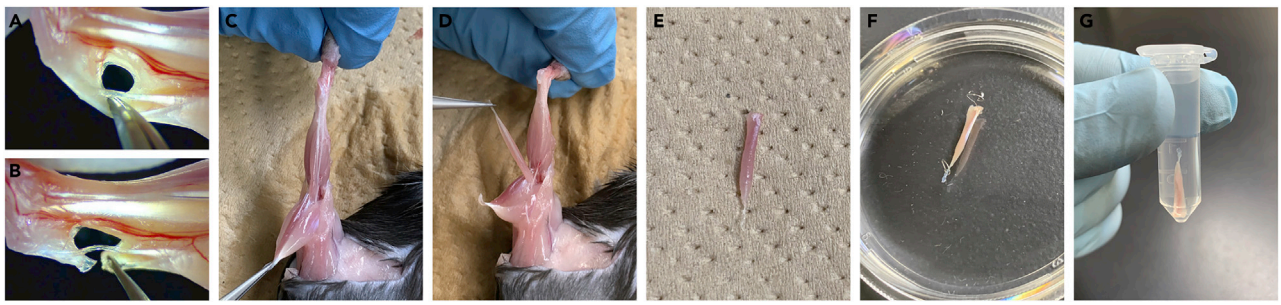

Figure 1. Excision and fixation of mouse plantaris muscle

(A and B) Cutting the Achilles tendon of the gastrocnemius and soleus muscles while leaving the Achilles tendon of the plantaris muscle intact.
(C) Exposure of the plantaris muscle.
(D) Cutting the Achilles tendon of the plantaris muscle.
(E) Isolation of the whole plantaris muscle.
(F) Pinning and fixation of the plantaris muscle.
(G) The fixed muscle in the tube.

Note: Use heterozygous PDGFRα^{EGFP} mice because homozygous for this knock-in allele is an embryonic lethal.

Note: In PDGFRα^{EGFP} mice, the H2B-eGFP fusion gene is expressed from the endogenous *Pdgfra* locus and mimics the expression pattern of the endogenous gene. Therefore, these mice are useful for the detection of PDGFRα(+) mesenchymal progenitors.

Fixation of mouse plantaris muscle

Timing: 30 min

7. Pour 4% PFA solution to silicone rubber plate and keep it on ice.
8. Immerse the plantaris muscle in 4% PFA solution and pin both ends of plantaris muscle using intradermal needles as quickly as possible (Figure 1F). See troubleshooting: problems 1 and 2.
9. Fix the muscle for 30 min on ice.
10. After fixation, transfer the muscle to a 2.0 mL tube containing PBS (Figure 1G).
11. Wash the muscle with PBS for 30 min three times.

⚠ CRITICAL: Pinning is required to preserve shape of muscle because muscles tend to curve if pinning is not performed during PFA fixation. See troubleshooting: problem 1.

Note: For pinning the end of plantaris muscle, fine-tipped forceps are recommended.

Pause point: The fixed muscle can be stored in PBS at 4°C for up to 1 week.

Blocking and whole-mount immunofluorescence staining

Timing: 3 days

The main purpose of this step is to stain whole plantaris muscle using antibody specific for PDGFRα to visualize mesenchymal progenitors. PDGFRα is specifically expressed on the surface of mesenchymal progenitors and is a useful marker for the detection of mesenchymal progenitors (Uezumi et al., 2010).

12. Remove PBS from the tube, add blocking solution to the tube, and then incubate the muscle in blocking solution (e.g., 500–600 μL) at 4°C for 12 h. See troubleshooting: problem 3.

Note: Use enough volume of blocking solution to completely soak the muscle.

13. Remove blocking solution from the tube, add goat polyclonal anti-mouse PDGFRα (final antibody concentration: 2.5 μg/mL) and rabbit monoclonal anti-YAP (1:100) diluted in blocking solution (e.g., 500–600 μL) to the tube, and incubate the muscle at 4°C for 12 h. A negative control sample that is not stained with the primary antibodies should also be prepared. See troubleshooting: problems 3 and 4.

⚠ CRITICAL: For whole-mount immunostaining, it is important to use a buffer containing a surfactant so that the antibody can access the antigen efficiently. We use blocking solution containing 1% Triton X-100 as an antibody diluent buffer. See troubleshooting: problems 2 and 3.

Note: Use enough volume of primary antibody solution to completely soak the muscle.

14. Wash the muscle with PBS for 30 min three times.

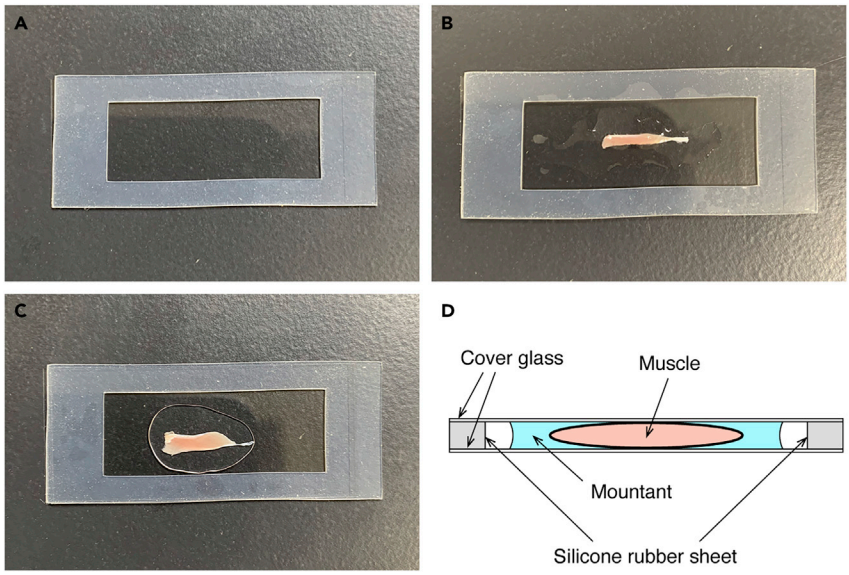

Figure 2. Mounting stained muscle for whole-mount imaging

(A) Cover glass with silicone rubber sheet.

(B) Muscle placed on the cover glass framed by silicone rubber sheet.

(C) Muscle sandwiched between cover glass.

(D) Schematic side cross-sectional view of mounted muscle.

15. Remove PBS from the tube, add Alexa 647-conjugated donkey anti-goat IgG and Alexa 594-conjugated donkey anti-rabbit IgG diluted in the blocking solution (1:1000 dilution) to the tube, and incubate the muscle at 4°C for 12 h in the dark. See troubleshooting: problems 3 and 4.
16. Remove secondary antibodies from the tube, add DAPI diluted in blocking solution (1:5000 dilution) to the tube, and incubate the muscle at 4°C for 10 min in the dark.
17. Wash the muscle with PBS for 30 min three times.

Note: Use enough volume (e.g., 500–600 μL) of secondary antibody and DAPI solution to completely soak the muscle.

Note: After secondary antibody staining, the sample should be protected from light.

Pause point: The stained muscle can be stored in PBS for up to 1 week at 4°C protecting from light.

Mounting stained muscle for confocal microscopy

Timing: 10 min/muscle

18. Place the silicone rubber sheet on cover glass (Figure 2A).
19. Place the stained muscle on a cover glass and put a few drops of mounting medium on the muscle (Figure 2B).

Note: We use SlowFade Diamond Antifade Mountant from Thermo Fisher Scientific but a similar antifade reagent can be used.

20. Cover the sample with another cover glass (Figure 2C).

Note: Sandwiching the sample between cover glasses from the top and bottom enables microscopic imaging from both sides (Figure 2D).

Pause point: The mounted specimen can be stored at 4°C protecting from light for up to 2 weeks.

Confocal microscopy

Timing: depending on number of samples

The main purpose of this step is to obtain z-stack images of the immunofluorescently stained whole muscle. For this purpose, a confocal laser scanning microscope is required. We only describe a general procedure for confocal microscopy here because detailed settings vary depending on the microscope used. Follow the manufacturer's instructions for detailed settings of each microscope.

21. Adjust the laser power and detector gain to obtain the appropriate signal in each channel while using a negative control sample for the background settings.
22. Set the start and end positions for z-stack imaging and determine the scan interval in the z-axis direction considering the optical resolution of the objective lens.

⚠ CRITICAL: For whole-mount observation, it is critical to use an objective lens with a working distance that adequately covers the depth of the sample to be observed.

Note: We use a 40× oil-immersion objective lens but other magnification lenses can be used depending on the imaging purpose.

Note: When taking z-stack images, first set the laser intensity to a sufficient level at the deepest part of the sample. Next, as you move the focus to the surface of the sample, check that the signal is not saturated at all depths. Some microscopes allow the laser intensity to be set in steps according to the sample depth, so it is useful to use this function.

Note: In z-stack imaging, the sample is repeatedly exposed to excitation lasers and is therefore prone to fading. It is recommended to test the degree of fading of the fluorescent dyes in the sample using test sample.

Note: The end position should be set at a depth of less than 50 μm from the muscle surface. See limitations.

23. Acquire the z-stack images. See troubleshooting: problem 5.
24. Using an appropriate 3D imaging software, reconstruct the maximum intensity projection image.

Note: We use the Leica TCS SP8 confocal laser scanning microscope system and the Leica LAS X software, but other confocal microscopes and the supplied 3D imaging software can also be used.

EXPECTED OUTCOMES

The described method allows a visualization of mesenchymal progenitors in murine plantaris muscle. As PDGFRα^{EGFP} mice express an enhanced green fluorescent protein gene fused to human histone H2B under the control of the endogenous *Pdgfra* promoter, the EGFP signal must be

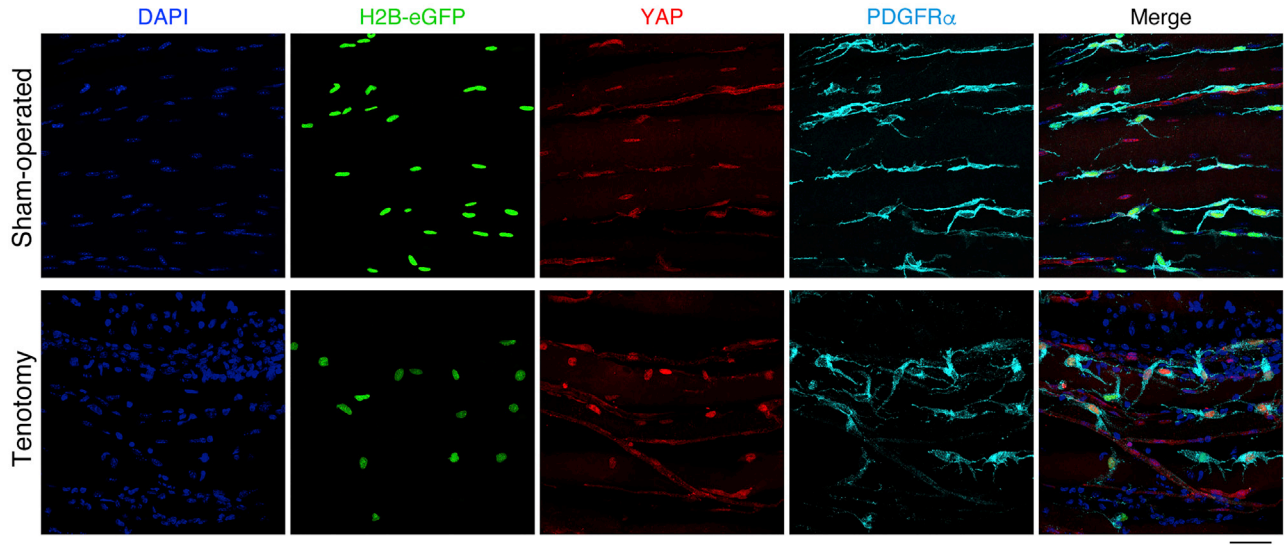

Figure 3. Representative image of whole-mount immunofluorescence staining of mesenchymal progenitors

Two days after operation, murine plantaris muscle was immunostained for YAP (red), PDGFRα (Cyan), and counterstained with DAPI (blue). H2B-eGFP (green) was not immunostained and native fluorescence was detected. Upper panels show sham-operated muscle and lower panels show muscle subjected to tenotomy. Scale bar: 50 μm.

detected in the nuclei of PDGFRα(+) cells (Figure 3). On the other hand, PDGFRα staining must be localized at the cell membrane (Figure 3).

Because YAP translocates to the nucleus in response to mechanical stimuli, a clear nuclear localization of YAP should be observed in mesenchymal progenitors of overloaded muscle (Figure 3 lower panels). In contrast, YAP should be localized primarily in the cytoplasm in mesenchymal progenitors of sham-operated muscle (Figure 3 upper panels).

LIMITATIONS

With the protocol presented here, it is difficult to obtain an adequate fluorescent signal from a point deeper than 50 μm from the muscle surface. Although sandwich covering with cover glasses allows microscopic observation from both sides of the muscle, there are substantial portions deeper than 50 μm from the surface in murine plantaris muscle. Therefore, if imaging deeper than 50 μm is required, tissue clearing should be performed.

TROUBLESHOOTING

Problem 1

Muscles curve during fixation and exhibit an unnatural shape (before you begin, step 1; step-by-step method details, step 8).

Potential solution

Muscles begin to curve after they are immersed in PFA solution. If the muscles are left in PFA solution without pinning, they are fixed in an unnatural curved form. Pinning both ends of plantaris muscle on silicone rubber plate prevents this artificial change in shape. Therefore, it is important to pin the end of muscle as quickly as possible before the muscle begins to curve.

Problem 2

Nuclear translocation of YAP is also observed in mesenchymal progenitors of sham-operated muscle (step-by-step method details, step 8).

Potential solution

The stimulus to isolate the muscle may also cause YAP activation, so excision and fixation of muscle should be performed as quickly as possible. Well-skilled experimenter can fix the muscle within 3 min after sacrificing the mouse.

Problem 3

Stained muscle shows high background (before you begin, step 3; step-by-step method details, steps 12, 13, and 15).

Potential solution

It is recommended to use high quality BSA for the blocking solution to reduce background staining. We use fatty acid-free and globulin-free grades of BSA for the blocking solution because these factors could cause nonspecific staining. Therefore, an equivalent grade of BSA should be used as the blocking reagent. In addition, as muscle tissues tend to produce a green autofluorescence, it is recommended to select fluorescent dye with higher wavelength such as Alexa-594 or Alexa 647 for secondary staining to obtain images with high S/N ratio.

Problem 4

PDGFRα or YAP staining is weak or unclear (before you begin, step 3; step-by-step method details, steps 13 and 15).

Potential solution

Insufficient penetration of antibody results in unclear staining. Using a buffer containing detergent such as 1% Triton X-100 as an antibody diluent is recommended to promote access of the antibody to the antigen. Antibody incubation time can be extended up to 2 days to enhance staining efficiency. It is also recommended to use secondary antibody conjugated with bright fluorescent dye (e.g., Alexa-594 or Alexa 647).

Problem 5

As the depth of the observation plane increases, the fluorescence signal becomes unclear (step-by-step method details, step 23).

Potential solution

An objective lens with a short working distance is not suitable for observing deeper portions. Objective lenses with a working distance greater than 0.2 mm are recommended for this protocol. In general, there is a trade-off between the numerical aperture (resolution) and the working distance of an objective lens. The balance between these two factors should be determined by carefully considering what is important in the image to be acquired (resolution or depth?).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Akiyoshi Uezumi (uezumi.akiyoshi@tokushima-u.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

A.K., A.U., and S.F. designed the experiments. T.K. and M.I.-U. performed experiments. A.U. and S.F. wrote the manuscript. T.K. and A.U. revised the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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