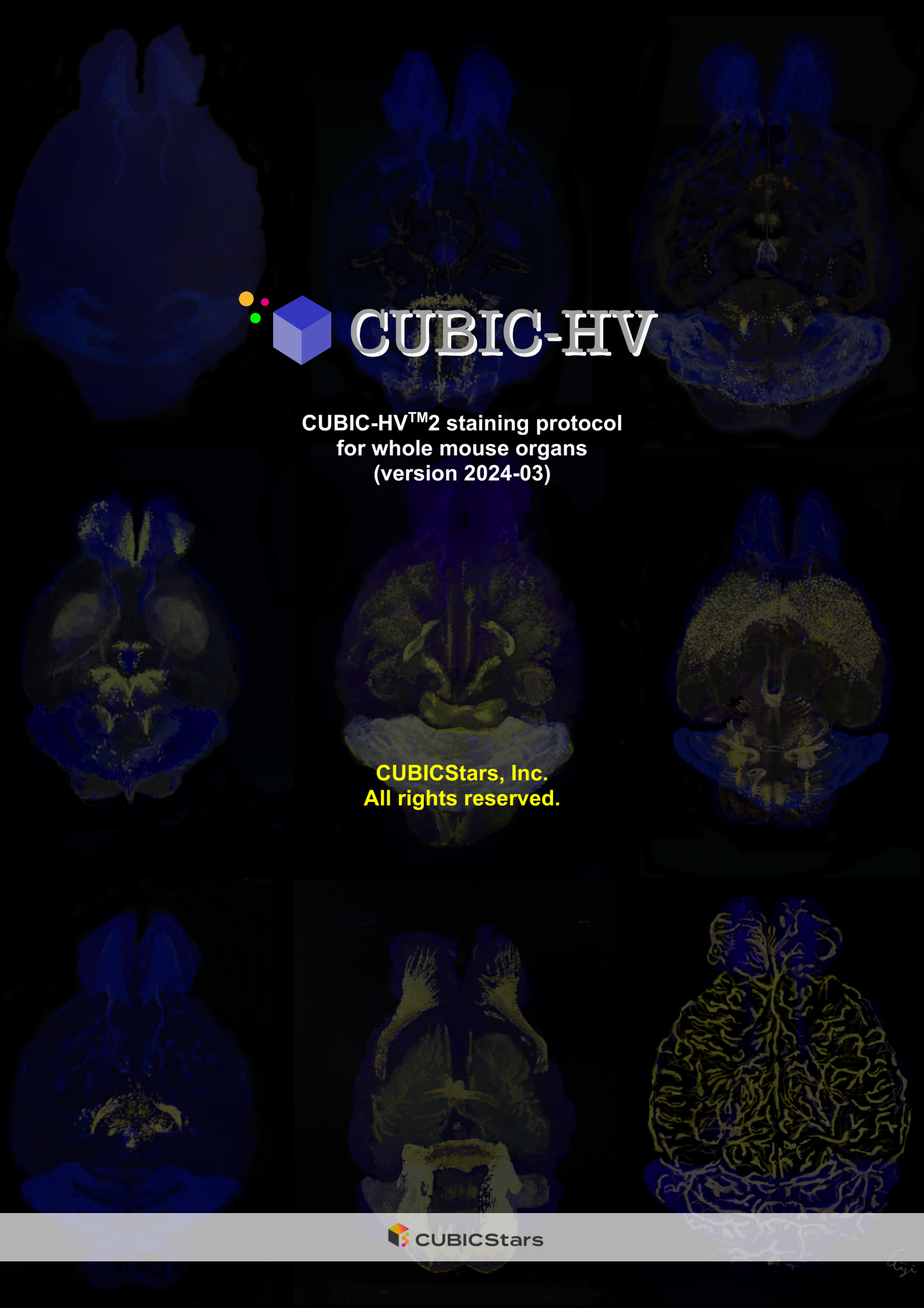


CUBIC-HV

CUBIC-HV™2 staining protocol
for whole mouse organs
(version 2024-03)

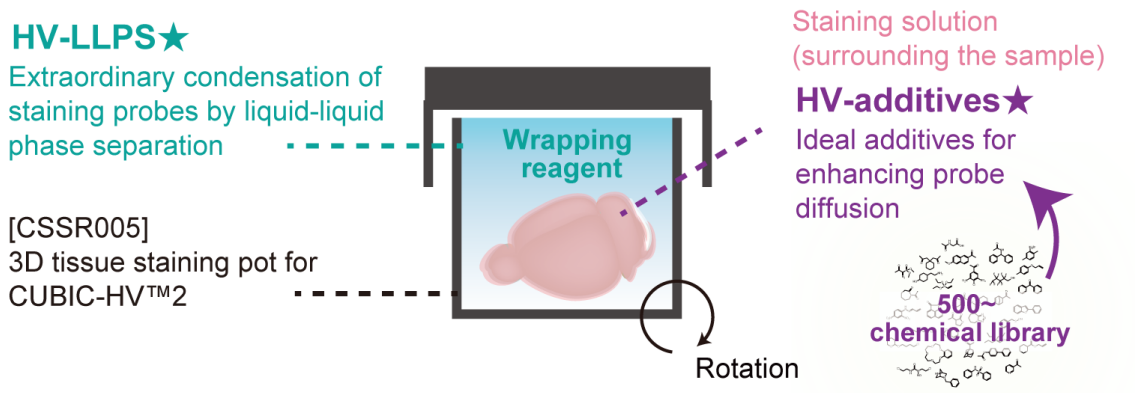
Product outline

Histological staining of three-dimensional volumetric tissue samples can be highly challenging. This is due to complications associated with the deep penetration of staining agents and antibodies within a volumetric tissue sample. CUBIC-HistoVIsion[2] has enabled versatile three-dimensional staining by resolving these issues. Important factors for 3D staining were identified in the research: 1) the concentration of the staining probe in the buffer, and 2) the appropriate modulation of probe-tissue interactions.

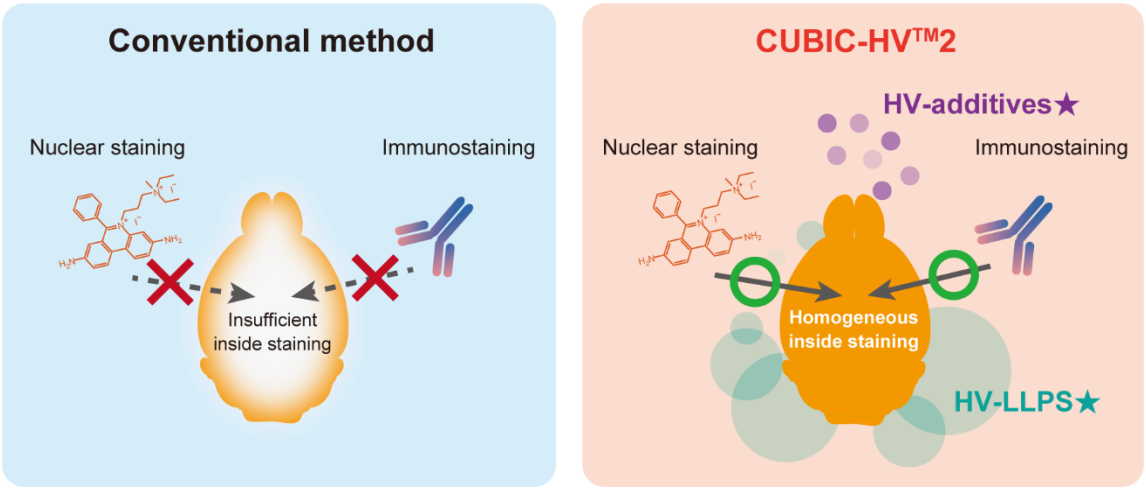
Based on the most recent version of the CUBIC-HistoVIsion protocol[1], our patented product, **CUBIC-HV™2**, integrates two innovative technologies to address these critical factors:

HV-LLPS★ is an innovative technique that increases the staining probe concentration through the reduction of the staining buffer volume to a minimum degree. By significantly condensing the staining probes (**50-100 µg/mL** for each primary antibody), this technology permits the deep staining of three-dimensional tissues in an efficient manner.

HV-additive★ is a set of ideal compounds that significantly enhance staining probe penetration, screened from a library of more than 500 compounds. By modulating probe-tissue interactions, deep diffusion of staining probes into three-dimensional tissues is strongly supported.



CUBIC-HV™2 enables your three-dimensional staining experiments for a variety of purposes without requiring specialized equipment or reagents that are not readily accessible.



NOTE

Generally, performing 3D staining on large tissue samples poses a challenge, even for users well-versed in histological techniques. We strongly advise initiating the application of this method with a benchmark experiment, specifically conducting anti-NeuN immunostaining with SYTOX-G nuclear staining on an entire mouse brain. Should you encounter any difficulties, please do not hesitate to reach out to our expert team for assistance. We also welcome any feedback from customers to improve this instruction.

Inquiry to our experts

JN EN CH

Examples & Technical tips

JN EN CH

Kit components

1× CUBIC-HV™2 Pre-wash buffer (150 mL)
2× CUBIC-HV™2 Staining buffer (80 mL)
10× CUBIC-HV™2 Additive A (16 mL)
10× CUBIC-HV™2 Additive B (16 mL)
10× CUBIC-HV™2 Additive C (16 mL)
10× CUBIC-HV™2 Additive D (16 mL)
10× CUBIC-HV™2 Additive E (16 mL)
10× CUBIC-HV™2 Additive F (16 mL)
CUBIC-HV™2 Wrapping reagent (35 mL)
2× CUBIC-HV™2 Wash buffer (300 mL)
Casein Sodium (0.8 g)*

*The day prior to usage, dissolve casein in 2× CUBIC-HV™2 Staining buffer through robust stirring using a stirrer.
■ No heating is necessary.
■ Avoid adding all the casein simultaneously; instead, introduce it gradually in multiple portions. Adding it all at once may lead to casein aggregation, resulting in incomplete dissolution.
■ Do not employ a vortex, as the buffer contains a high concentration of detergent, which could lead to foaming.

Additional reagents to be used

PBS (Tablet): TaKaRa #T9181
Sodium azide (NaN_3): Nacalai tesque #31208-82
Paraformaldehyde (PFA): Nacalai tesque #02890-45
Formalin solution: Nacalai tesque #16222-65
Heparin: FUJIFILM Wako #081-00136
Tissue-Clearing Reagent CUBIC-L: CUBICStars #CSCR001
Tissue-Clearing Reagent CUBIC-R+(N): CUBICStars #CSCR002

Recommended nuclear stains

Choose a suitable nuclear stain for your experimental design:

- Blue-green: BOBO™-1 Iodide (462/481), ThermoFisher Scientific #B3582
  Can be used with yellow-orange (e.g., AF532, Cy3, tdTomato), orange-red (e.g., AF594, mCherry), and red-far red (e.g., AF647) dyes and fluorescent proteins

- Green: SYTOX™ Green Nucleic Acid Stain, ThermoFisher Scientific #S7020
  Can be used with orange-red (e.g., AF594, mCherry), and red-far red (e.g., AF647) dyes and fluorescent proteins

- Red-far red: RedDot™2 Far-Red Nuclear Stain, biotium #40061
  Can be used with green (e.g., FITC, GFP, YFP), yellow-orange (e.g., AF532, Cy3, tdTomato), and orange-red (e.g., AF594, mCherry) dyes and fluorescent proteins

Antibodies

Primary antibody
■ It is recommended to use antibody products containing a concentration of 250 µg/mL or higher.

Secondary antibody
FabuLight™: Fc specific Fab fragment (Jackson Immunolab)
https://www.jacksonimmuno.com/catalog/31#target:15
■ Alexa Fluor® 488 is quenched in CUBIC-R. We recommend FabuLight™ products labeled with Cy3, Alexa Fluor® 594, or Alexa Fluor® 647.
■ Alternatively, direct dye-conjugated primary antibodies may be employed. The secondary antibody is unnecessary in this case.

Containers to be used

Protein LoBind 500 µL tube: Eppendorf #022431064
5 mL tube: Eppendorf #0030119401
15 mL standing tube: SARSTEDT #60.732.001
30 mL tube: SARSTEDT #60.544
50 mL tube: Falcon #352070
3D tissue staining pot for CUBIC-HV™2: CUBICStars #CSSR005

Process outline

Perfusion fixation and dissection of the mouse organ

↓

Post-fix

↓ 18~24 h at 4°C

Wash (PBS)

↓ 2 h ×3 at RT

Delipidation (CUBIC-L)

↓ 3~7 days at 37°C

Wash (PBS/NaN_3)

↓ 2 h ×3 at 37°C → (Can be stored at 4°C)

Pre-wash (CUBIC-HV™2 Pre-wash buffer)

↓ overnight at 37°C

Primary antibody + secondary Fab reaction

Exchange to the CUBIC-HV™2 Staining buffer containing HV-additives★

↓ 1.5 h at 37°C

Nuclear & immunostaining (CUBIC-HV™2 Staining buffer with HV-LLPS★ & HV-additives★)

↓ 1 week~ at RT

Reaction at 4°C

↓ 1 day

Wash (CUBIC-HV™2 Wash buffer)

↓ 1.5 h ×3 at 4°C

Post-fix (1% FA in CUBIC-HV™2 Wash buffer)

↓ 24 h at 4°C → 1 h at 37°C

Wash (PBS)

↓ 2 h at RT

RI matching (CUBIC-R+)

↓ 50% diluted CUBIC-R+ overnight at >25°C

↓ 100% CUBIC-R+ 2 days~ at >25°C

(Gel embedding)

↓

Microscopic observation

Process details

*** The reaction time and quantity of reagent required to stain a single whole mouse brain are detailed in this protocol.**

■ With the exception of staining and the 4°C reaction, every shaking phase is executed in the horizontal orientation.

■ Delipidated organ samples are susceptible to injury and should be handled with a metal spoon.

1) Collection of mice organs[3,4]

1. Anesthetize the mouse.
2. Transcardially perfuse 10 mL (4 mL/min) of ice-cold PBS containing 10 U/mL of Heparin.
3. Transcardially perfuse 20 mL (6 mL/min) of ice-cold 4% (w/v) PFA.
4. Dissect the organs.
5. Post-fix the dissected organs in 4% (w/v) PFA in PBS (~ 10 mL/whole organ) for overnight (8-24 h) at 4°C with gentle shaking (40-50 rpm/min).
6. Wash the sample in PBS (+0.05% NaN_3) for 3 h × 3 times at room temperature with gentle shaking (40-50 rpm/min).

2) Delipidation with CUBIC-L[3]

1. Immerse the fixed sample in 10 mL of 0.5× CUBIC-L (1:1 dilution with water) in the 30 mL tube and incubate it for overnight at 37°C with gentle shaking (40-50 rpm/min).
2. Replace to 10-15 mL of 1× CUBIC-L in the 30 mL tube and delipidate for 3-5 days at 37°C with gentle shaking (40-50 rpm/min).

■ Replace CUBIC-L every 2 to 3 days if the duration of treatment exceeds 3 days.

3. Wash the sample with 20 mL of PBS containing 0.05% NaN_3 for 2 h × 3 times (or 2 h ×1, overnight ×1, 2 h ×1) at 37°C with gentle shaking (40-50 rpm/min).

■ After each use, the tubes should be washed or replaced in order to remove Triton X-100 thoroughly.

■ The delipidated sample can be stored in PBS/0.05% NaN_3 at 4°C at least for several months.

3) 3D staining with HV-LLPS★ and HV-additives★

1. Immerse the delipidated sample in 10 mL of 1× CUBIC-HV™2 Pre-wash buffer in the 15 mL standing tube and incubate it for overnight at 37°C with gentle shaking (40-50 rpm/min).
2. Exchange the buffer to 10 mL of 1× CUBIC-HV™2 Staining buffer containing selected 1× HV-additive★ reagents and incubate it for 1.5 h~ at 37°C with gentle shaking (40-50 rpm/min).

■ The type of HV-additives★ should be determined for each 1st antibody. Generally, one HV-additives★ reagent or two are used.

For 10 mL of the 1× CUBIC-HV™2 Staining buffer containing a single HV-additive★ reagent:

2× CUBIC-HV™2 Staining buffer	5 mL (final 1×)
10× HV-additives★ reagent	1 mL (final 1×)
Distilled water	4 mL
Total	10 mL

3. During the step 2, prepare 1st Ab + 2nd Fab fragment mix as follows.
   a. Calculate the required volume (X, Y) of 1st Ab and 2nd Fab fragment.
      1st Ab: 1-5 μg/whole mouse brain (approximately 0.6 g)
      In the case of using 5 μg of 1 mg/mL (= 1 μg/μL) 1st Ab product, X = 5 (μg) /1 (μg/μL) = 5 μL.
      2nd Fab fragment: 4 μg/whole mouse brain
      In the case of using 1.5 mg/mL product, Y = 4 (μg)/1.5 (μg/μL) ≒ 2.67 μL.

■ When using a direct dye-conjugated primary antibody, 3-a can be skipped.

■ The amount and the final concentration of 1st Ab should be determined for each application.

■ When applying to organs other than the brain, estimate the required quantity of antibodies and reagents by considering the weight ratio of the organ.

b. Prepare the following 3D staining solution (typically 50-100 µL per whole mouse brain).

■ The total volume of the staining solution should be determined based on the quantity and final concentration of 1st Ab. For instance, in the case of employing 5 µg of 1st Ab at a concentration of 100 µg/mL, a volume of 50 µL should be prepared for the 3D staining solution (5 µg of 1st Ab in 50 µL = 100 µg/mL). Generally, 50-100 µg/mL of antibody concentration prepared in 50-350 µL of the staining solution is recommended.

■ The type of HV-additives★ should be determined for each 1st antibody (the guide is provided on our website). Generally, one HV-additives★ reagent or two are used.

For 100 µL of the 3D staining solution containing a single HV-additive★ reagent:

2× CUBIC-HV™2 Staining buffer	50 µL (final 1x)
1st Ab	X µL
2nd Fab fragment	Y µL
Nuclear stain*	Z µL
10× HV-additives★ reagent	10 µL
Distilled water	50 - (X+Y+Z+10) µL
Total	100 µL

Add the entire volume to the 500 µL Protein LoBind tube and incubate the mix for 1.5 h at 37°C.

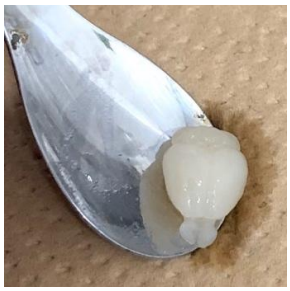
*Recommended amount of nuclear stain
BOBO™-1 Iodide (462/481): 4 µL/whole mouse brain
SYTOX™ Green Nucleic Acid Stain: 1.6 µL/whole mouse brain
RedDot™2 Far-Red Nuclear Stain: 4 µL/whole mouse brain

4. After the reaction in step 3, transfer the entire 3D staining solution into the 3D tissue staining pot [CSSR005].

■ This 3D tissue staining pot for CUBIC-HV™2 can be used repeatedly. After the usage, wash the pot with a neutral detergent and distilled water.

5. Collect the sample with a metal spoon and put it into the staining pot.

■ A portion of the sample should be attached gently on soft paper towel to absorb residual buffer.



6. Slowly pour CUBIC-HV™2 Wrapping reagent into the staining pot until the pot is filled. Remove the bubbles.

■ The wrapping reagent prevents samples from drying out during staining by covering the entire sample and the staining solution containing condensed staining probes (HV-LLPS★).

7. Close the lid tightly and incubate the chamber for 1 week~ at RT with slow rotation (<1 rpm) under a light-shielded condition.

■ **The staining period should be determined for each antibody.**

8. To stabilize the 2nd Fab signals, further incubate the staining chamber for 24 h at 4°C with slow rotation (<1 rpm) under a light-shielded condition.
9. 1 h before moving to the step 10, prepare 1× CUBIC-HV™2 Wash buffer and cool it on ice.

For 40 (10 × 4) mL of the 1× CUBIC-HV™2 Wash buffer:

2× CUBIC-HV™2 Wash buffer	20 mL (final 1×)
Distilled water	20 mL
Total	20 mL

10. Recover the sample from the staining pot with a metal spoon. Discard the staining solution and the wrapping reagent.
11. Remove the residual buffer and the wrapping reagent as in step 5.
12. Immerse the sample in 10 mL of pre-cooled 1× CUBIC-HV™2 Wash buffer in the 15 mL standing tube. Gently invert the tube several times to separate the wrapping reagent remaining on the sample surface.
13. Replace the 1× CUBIC-HV™2 wash buffer with a new one and continue washing the sample gently at 4°C with light shielding, while shaking at 40-50 rpm/min, for 2 h.
14. Replace the wash buffer with the new one and continue washing the sample two more times (2 h ×2).

4) Post-fix with 1% FA

1. During the sample wash, prepare 7~8 mL of fixative solution by diluting formalin (FA) solution to final 1% in 1× CUBIC-HV™2 Wash buffer and cool it on ice.

■ **The saturated formalin solution contains 35 to 38% of formalin. For example, when using a 37% formalin solution, dilute it with 1x CUBIC-HV™2 Wash buffer at a ratio of 1:36.**

For 7.4 mL of the FA solution:

2× CUBIC-HV™2 Wash buffer	3.7 mL (final 1×)
Saturated formalin (37%)*	0.2 mL (final 1%)
*The concentration may be different by products.	
Distilled water	3.5 mL
Total	7.4 mL

2. Fix the sample in the 1% FA for 24 h at 4°C with gentle shaking (40-50 rpm/min) under a light-shielded condition.
3. To accelerate the fixation reaction, further incubate the sample for 1 h at 37°C with gentle shaking (40-50 rpm/min) under a light-shielded condition.

■ **Reducing the FA reaction time in steps 2 and 3 could result in the attenuation of antibody signals.**

4. Wash the sample in 30 mL of PBS in the 50 mL tube for 2 h at RT with gentle shaking (40-50 rpm/min) under a light-shielded condition.

5) RI matching with CUBIC-R+

1. Incubate the sample in 15 mL of 0.5× CUBIC-R+ (1:1 diluted with distilled water) in a 30 mL tube for 24 h at >25°C with gentle shaking (40-50 rpm/min) under a light-shielded condition.
2. Remove the residual diluted CUBIC-R+ as in section 3-step 5 and incubate the sample in 15 mL (or 30 mL if gel embedding is applied) of 1× CUBIC-R+ for 2 days at >25°C with gentle shaking (40-50 rpm/min) under a light-shielded condition.

■ Carryover of residual diluted CUBIC-R+ results in a poor clearing result due to a decrease in the final refractive index.

■ CUBIC-R+ crystallizes easily in winter. Ensure that sample in the reagent are handled and incubated at 25°C or higher.

■ If required, CUBIC-R-cleared samples can be embedded in 2% agarose/CUBIC-R. For more details, please refer to the original paper (ref. 3) and RIKEN CUBIC resource website (http://cubic.riken.jp/)

3. Use the sample for microscopic observation (gel embedding if necessary)

CUBIC-HV™2
Guide for evaluating primary antibodies & HV-additive★

Reagents to be used

PBS (Tablet): TaKaRa #T9181
Sodium azide (NaN_3): Nacalai tesque #31208-82
Paraformaldehyde (PFA): Nacalai tesque #02890-45
Formalin solution: Nacalai tesque #16222-65
Heparin: FUJIFILM Wako #081-00136
Sucrose: Nacalai tesque #30403-55
DAPI (1 mg/mL solution): Thermo Fisher #62248
Tissue-Clearing Reagent CUBIC-L: CUBICStars #CSCR001

2× CUBIC-HV™2 Staining buffer
10× CUBIC-HV™2 Additive A
10× CUBIC-HV™2 Additive B
10× CUBIC-HV™2 Additive C
10× CUBIC-HV™2 Additive D
10× CUBIC-HV™2 Additive E
10× CUBIC-HV™2 Additive F
2× CUBIC-HV™2 Wash buffer
Casein Sodium*
*The day prior to usage, dissolve casein in 2× CUBIC-HV™2 Staining buffer through robust stirring using a stirrer, according to the CUBIC-HV™2 general protocol.

Cryopreservation reagent
40(w/v)% Sucrose (100 mL)：dissolve 40 g of sucrose in PBS and adjust the total volume to 100 mL.
25(w/v)% Sucrose (100 mL)：dissolve 25 g of sucrose in PBS and adjust the total volume to 100 mL.

Antibodies

Primary antibody
■ It is recommended to use antibody products containing a concentration of 250 μg/mL or higher.

Secondary antibody
FabuLight™: Fc specific Fab fragment (Jackson Immunolab)
https://www.jacksonimmuno.com/catalog/31#target:15
■ Alexa Fluor® 488 is quenched in CUBIC-R. We recommend FabuLight™ products labeled with Cy3, Alexa Fluor® 594, or Alexa Fluor® 647.
■ Alternatively, direct dye-conjugated primary antibodies may be employed. The secondary antibody is unnecessary in this case.

Containers to be used

Protein LoBind 500 μL tube: Eppendorf #022431064
15 mL standing tube: SARSTEDT #60.732.001
3D tissue staining pot for CUBIC-HV™2: CUBICStars #CSSR005
24 well plate：Falcon #353047

Process outline

Perfusion fixation and dissection of the mouse organ

↓

Post-fix

↓ 18~24 h at 4°C

Wash (PBS)

↓ 2 h ×3 at RT

Delipidation (CUBIC-L)

↓ 3~7 days at 37°C

Wash (PBS/NaN_3)

↓ 2 h ×3 at 37°C → (Can be stored at 4°C)

Prepare cryosections

↓ wash → (Can be stored at 4°C)

STEP 1: Evaluate primary antibodies (2D staining)

STEP 2: Evaluate suitable HV-additives★ for each primary antibody (2D staining)

STEP 3: Determine 3D staining condition for each primary antibody

↓

Prepare cryosections

Process details

< Sample preparation >

1) Collection of mice organs[3,4]

1. Anesthetize the mouse.
2. Transcardially perfuse 10 mL (4 mL/min) of ice-cold PBS containing 10 U/mL of Heparin.
3. Transcardially perfuse 20 mL (6 mL/min) of ice-cold 4% (w/v) PFA.
4. Dissect the organs.
5. Post-fix the dissected organs in 4% (w/v) PFA in PBS (~ 10 mL/whole organ) for overnight (8-24 h) at 4°C with gentle shaking (40-50 rpm/min).
6. Wash the sample in PBS (+0.05% NaN_3) for 2 h × 3 times at room temperature with gentle shaking (40-50 rpm/min).

2) Delipidation with CUBIC-L[3]

1. Immerse the fixed sample in 10 mL of 0.5× CUBIC-L (1:1 dilution with water) in the 30 mL tube and incubate it for overnight at 37°C with gentle shaking (40-50 rpm/min).
2. Replace to 10-15 mL of 1× CUBIC-L in the 30 mL tube and delipidate for 3-5 days at 37°C with gentle shaking (40-50 rpm/min).

■ **Replace CUBIC-L every 2 to 3 days if the duration of treatment exceeds 3 days.**

3. Wash the sample with 20 mL of PBS containing 0.05% NaN_3 for 2 h × 3 times (or 2 h ×1, overnight ×1, 2 h ×1) at 37°C with gentle shaking (40-50 rpm/min).

■ **After each use, the tubes should be washed or replaced in order to remove Triton X-100 thoroughly.**

■ **The delipidated sample can be stored in PBS/0.05% NaN_3 at 4°C at least for several months.**

3) Cryosection of CUBIC-L-treated tissues

1. For cryopreservation, immerse the CUBIC-L-treated sample in 40% (w/v) sucrose/PBS and maintain it at 4°C until the sample settles at the bottom of the tube (typically it takes over several hours to overnight).
2. Also, for cryopreservation of the fixed sample (without CUBIC-L treatment), immerse the sample in 10% (w/v) sucrose/PBS and keep at 4°C until the sample settles at the bottom of the tube. Subsequently, exchange the reagent to 25% (w/v) and maintain it at 4°C until the sample settles again. Typically, overnight incubation will be needed.
3. Embed the samples in O.C.T. compound.
4. Use a cryostat to prepare sections of these samples, cutting them at a thickness of 50 μm (for CUBIC-L-treated samples) or 30 μm (for fixed samples). Gather and wash the sections in PBS.

■ **The prepared sections can be stored in PBS/0.05% NaN_3 at 4°C for at least several months.**

< Evaluate primary antibodies and HV-additives★ >

STEP 1: Evaluate primary antibodies (2D staining)

In this step, the compatibility of primary antibodies with CUBIC-L-treated tissue samples is evaluated. Cryosectioned tissue samples are stained with a selected primary antibody in the CUBIC-HV™2 Staining buffer using a 24-well plate. It is advisable to compare the staining patterns between fixed tissue sections (without CUBIC-L treatment) and sections from CUBIC-L-treated samples, as the CUBIC-L treatment may alter antigenicity. Additionally, the CUBIC-L treatment may have an antigen retrieval effect and occasionally enhances the immunostained signals.

Procedures:

1. Calculate the required amount (X, Y) of primary and secondary (Fab fragment) antibodies.
   - **■ A total of 250 µL of staining solution is used per well to stain a single slice in a 24-well plate.**
   - **■ Initiating the dilution rate of any primary antibodies at 1/100 or 1/1000 is recommended.**

   For example, when using the 1 µg/µL of primary antibody product and the 1.5 µg/µL of secondary Fab fragment product:
   X (volume) = 2.5 µL of the primary antibody (1/100 dilution) /well
   X (amount) = 2.5 (µL) × 1.0 (µg/µL) = 2.5 µg /well
   Y (volume) = 2.5 (µg) / 1.5 (µg/µL) ≃ 1.67 µL of the secondary Fab fragment/well
   (primary : secondary = 1 : 1 in weight ratio)

2. Mix X µL of primary antibody and Y µL of secondary antibody in a Protein LoBind 500 µL tube. Incubate it protected from light for 1.5 h at 37°C.
   - **■ When using a direct dye-conjugated primary antibody, this can be skipped.**

3. Preparation of the antibody staining solution.

   For 1/100 dilution:

2× CUBIC-HV™2 Staining buffer	125 µL (final 1×)
Distilled water	125 - (X + Y) µL
Antibody complex	X + Y µL (final 1/100-diluted primary antibody)
==========	
Total	250 µL

   For 1/1000 dilution:

2× CUBIC-HV™2 Staining buffer	112.5 µL (final 1×)
Distilled water	112.5 µL
The above 1/100-diluted staining solution	25 µL*
==========	
Total	250 µL

   *Take a portion of the 1/100-diluted staining solution

4. Immerse a section in the antibody staining solution using a 24-well plate. Incubate it protected from light for 1 day at room temperature (approximately 25°C) with gentle shaking (40-50 rpm/min) using a table shaker.
■ Cover the wells with Parafilm to prevent evaporation of the antibody staining solution, and then place a lid on the 24-well plate.
5. Incubate it protected from light for 1 h at 4°C to stabilize the binding of the secondary Fab fragment.
6. During step 5, prepare 2 mL of 1× CUBIC-HV™2 Wash buffer with DAPI (1/1000) and cool it on ice.
■ For preparing 1× CUBIC-HV™2 Wash buffer, dilute 1 mL of 2× CUBIC-HV™2 Wash buffer in the kit with the same amount of distilled water.
7. Wash the stained section in 1 mL of the ice-cold 1× CUBIC-HV™2 Wash buffer with DAPI in a 24 well plate protected from light for 30 min x 2 times at 4°C with gentle shaking (40-50 rpm/min).
■ The wash buffer should be cooled at 4°C or on ice before use.
8. Replace the wash buffer to 1 mL of ice-cold PBS in a 24 well plate protected from light for 10 min at 4°C with gentle shaking (40-50 rpm/min).
■ PBS should be cooled at 4°C or on ice before use.

9. Place the sections on a slide glass for preparation and observed it with an epifluorescence microscope.
■ For optimal assessment and comparison of staining signals, it is advisable to capture a complete image of the preparation, incorporating all sections, utilizing a low-magnification objective (4X) and employing the tiling function of the microscopy system.

−If an appropriate staining signal is obtained, proceed to STEP 2.
■ Choose an appropriate dilution rate (1/100 or 1/1000) based on the outcome.

−If no or only a weak signal is obtained, or non-specific signals are observed, follow the troubleshooting as below:
■ The fixation method, such as PFA concentration or fixation time, should be considered. Try reduced PFA concentration (4% to 1 or 2%) and skip the post-fixation step.
■ CUBIC-HV™ does not include an antigen activation step since the CUBIC-L treatment may have a certain antigen-activating effect.
■ Staining at 4°C can occasionally improve the results.
■ If the above-mentioned optimizations fail to yield an acceptable result, the antibody should be excluded.

STEP 2: Evaluate suitable HV-additives★ for each primary antibody

HV-additives★ are our patented probe penetration enhancers, each with distinct stringencies. Selecting an appropriate additive or combination of additives is essential for the successful 3D staining using CUBIC-HV™2. This section details the process of evaluating a suitable HV-additive★ for a specific primary antibody.

Recommended HV-additive★ combinations for the initial evaluation:

1) No additive
2) 0.5× Additive A
3) 1× Additive A
4) 1× Additive A + 1× Additive B (or 2× Additive B for further enhancement)
5) 1× Additive B + 1× Additive C (or 2× Additive C for further enhancement)
6) 1× Additive A + 1× Additive B + 1× Additive D
7) 1× Additive B + 1× Additive C + 1× Additive D
8) 1× Additive E
9) 1× Additive F
10) 1× Additive A + 1x Additive F

■ Combinations 2) to 5) encompass most antibody cases. Users can initiate testing with these five conditions.
■ Additive D is used to further enhance antibody penetration in combinations 4), 5), and 10).
■ Combination 8) is recommended for antibodies exhibiting extreme resistance to penetration. It should be noted that the high stringency of this additive may significantly reduce antibody signals. In such cases, testing a more diluted condition (e.g., 0.5x additive E) is advisable.
■ Combinations 9) and 10) serve as optional alternatives and can be compared to combinations 3) to 7).

Procedures:

1. Calculate the required amount (X, Y) of primary and secondary (Fab fragment) antibodies.
   ■ A total of 250 µL of staining solution is used per well to stain a single slice in a 24-well plate.
   ■ Use the dilution rate (1/100 or 1/1000) of the primary antibody determined in STEP 1.

   For example, when using the 1 µg/µL of primary antibody product and the 1.5 µg/µL of secondary Fab fragment product at the dilution rate of 1/100:
   X (volume) = 2.5 µL of the primary antibody (1/100 dilution) /well
   X (amount) = 2.5 (µL) × 1.0 (µg/µL) = 2.5 µg /well
   Y (volume) = 2.5 (µg) / 1.5 (µg/µL) ≃ 1.67 µL of the secondary Fab fragment/well
   (primary antibody: secondary Fab fragment = 1 : 1 in weight ratio)

   In the case of dilution rate at 1/1000:
   First, prepare the 1/10-diluted antibody solutions (for testing all the 10 HV-additive★ combinations).
   ■ Modify the following recipes based on the number of tested HV-additive★ combinations.

Primary antibody	3 µL
Distilled water	27 µL
Total	30 µL (0.1 µg/µL of the primary antibody)

Secondary Fab fragment	2 µL
Distilled water	18 µL
Total	20 µL (0.15 µg/µL of the secondary Fab fragment)

   Then, calculate X and Y.
   X (volume) = 2.5 µL of the 1/10-diluted primary antibody (final 1/1000 dilution) /well
   X (amount) = 2.5 (µL) × 0.1 (µg/µL) = 0.25 µg /well
   Y (volume) = 0.25 (µg) / 0.15 (µg/µL) ≃ 1.67 µL of the 1/10-diluted secondary Fab fragment /well
   (primary antibody : secondary Fab fragment = 1 : 1 in weight ratio)

2. To test all 10 combinations simultaneously, mix 11 times X µL of primary antibody and 11 times Y µL of secondary antibody in a Protein LoBind 500 µL tube. Incubate the mixture protected from light for 1.5 h at 37°C.
   ■ **Adapt the mixing volume based on the number of combinations being tested.**
   ■ **When using a direct dye-conjugated primary antibody, this step can be skipped.**

3. Preparation of the antibody staining solutions 1)-10) containing HV-additive★.

1) No additive (control)

2× CUBIC-HV™2 Staining buffer	125 µL (final 1×)
Distilled water	125 - (X+Y) µL
Antibody complex	X + Y µL
Total	250 µL

2) 0.5x Additive A

2× CUBIC-HV™2 Staining buffer	125 µL (final 1×)
10× Additive A	12.5 µL (final 0.5×)
Distilled water	125 - (X+Y-12.5) µL
Antibody complex	X + Y µL
Total	250 µL

3) 1x Additive A

2× CUBIC-HV™2 Staining buffer	125 µL (final 1×)
10× Additive A	25 µL (final 1×)
Distilled water	125 - (X+Y-25) µL
Antibody complex	X + Y µL
Total	250 µL

4) 1x Additive A + 1x Additive B

2× CUBIC-HV™2 Staining buffer	125 µL (final 1×)
10× Additive A	25 µL (final 1×)
10× Additive B	25 µL (final 1×)
Distilled water	125 - (X+Y-50) µL
Antibody complex	X + Y µL
Total	250 µL

■ **Alternatively, 1× Additive A + 2× Additive B can be considered for further enhancement.**

5) 1x Additive B + 1x Additive C

2× CUBIC-HV™2 Staining buffer	125 µL (final 1×)
10× Additive B	25 µL (final 1×)
10× Additive C	25 µL (final 1×)
Distilled water	125 - (X+Y-50) µL
Antibody complex	X + Y µL
Total	250 µL

■ **Alternatively, 1× Additive B + 2× Additive C can be considered for further enhancement.**

6) 1x Additive A + 1x Additive B + 1x Additive D

2× CUBIC-HV™2 Staining buffer	125 µL (final 1×)
10× Additive A	25 µL (final 1×)
10× Additive B	25 µL (final 1×)
10× Additive D	25 µL (final 1×)
Distilled water	125 - (X+Y-75) µL
Antibody complex	X + Y µL
Total	250 µL

7) 1x Additive B + 1x Additive C + 1x Additive D

2× CUBIC-HV™2 Staining buffer	125 µL (final 1×)
10× Additive B	25 µL (final 1×)
10× Additive C	25 µL (final 1×)
10× Additive D	25 µL (final 1×)
Distilled water	125 - (X+Y-75) µL
Antibody complex	X + Y µL
Total	250 µL

8) 1x Additive E

2× CUBIC-HV™2 Staining buffer	125 µL (final 1×)
10× Additive E	25 µL (final 1×)
Distilled water	125 - (X+Y-25) µL
Antibody complex	X + Y µL
Total	250 µL

■ Alternatively, a diluted condition (e.g., 0.5x Additive E) can be considered when the antibody signals are significantly reduced.

9) 1x Additive F

2× CUBIC-HV™2 Staining buffer	125 µL (final 1×)
10× Additive F	25 µL (final 1×)
Distilled water	125 - (X+Y-25) µL
Antibody complex	X + Y µL
Total	250 µL

10) 1x Additive A + 1x Additive F

2× CUBIC-HV™2 Staining buffer	125 µL (final 1×)
10× Additive A	25 µL (final 1×)
10× Additive F	25 µL (final 1×)
Distilled water	125 - (X+Y-50) µL
Antibody complex	X + Y µL
Total	250 µL

4. Immerse a section in each of the antibody staining solutions using a 24-well plate. Incubate it protected from light for 1 day at room temperature (approximately 25°C) with gentle shaking (40-50 rpm/min) using a table shaker.

■ Cover the wells with Parafilm to prevent evaporation of the antibody staining solution, and then place a lid on the 24-well plate.

5. Incubate it protected from light for 1 h at 4°C to stabilize the binding of the secondary Fab fragment.

6. During step 5, prepare 2 mL of 1× CUBIC-HV™2 Wash buffer with DAPI (1/1000) and cool it on ice.

■ For preparing 1× CUBIC-HV™2 Wash buffer, dilute 1 mL of 2× CUBIC-HV™2 Wash buffer in the kit with the same amount of distilled water.

7. Wash the stained section in 1 mL of the ice-cold 1× CUBIC-HV™2 Wash buffer with DAPI in a 24 well plate protected from light for 30 min x 2 times at 4°C with gentle shaking (40-50 rpm/min).

■ The wash buffer should be cooled at 4°C or on ice before use.

8. Replace the wash buffer to 1 mL of ice-cold PBS in a 24 well plate protected from light for 10 min at 4°C with gentle shaking (40-50 rpm/min).

■ PBS should be cooled at 4°C or on ice before use.

9. Place the sections on a slide glass for preparation and observed it with a fluorescence microscope.

■ For optimal assessment and comparison of staining signals, it is advisable to capture a complete image of the preparation, incorporating all sections, utilizing a low-magnification objective (4X) and employing the tiling function of the microscopy system.

10. Compare the staining signals in each of the tested HV-additive★ combinations with **1) No additive** condition.

−Omit the conditions that 1) causes higher background signals, an/or 2) significantly reduce the staining signals.

< Determine the 3D staining condition for each primary antibody with HV-additives★ >

STEP 3: Determine 3D staining condition for each primary antibody

During this final step, the compatibility of the chosen HV-additive★ combinations in **STEP 2** for CUBIC-HV™2 3D staining is assessed. HV-LLPS★ is also integrated into this process. It is advisable to utilize a relatively small tissue block (e.g., a mouse brain hemisphere) for the evaluation. Once the optimal HV-additive★ combination is determined, the amount of the primary antibody is adjusted accordingly.

Procedures:

■ The reagent volumes specified here are based on the usage of a mouse brain hemisphere (0.3 g). Adjust the necessary volumes accordingly for other tested organs/tissues.

1. Immerse the delipidated sample (prepared through steps **1)** and **2)** of the above **<Sample preparation>** procedure) in 5 mL of 1× CUBIC-HV™2 Pre-wash buffer in the 15 mL standing tube and incubate it for overnight at 37°C with gentle shaking (40-50 rpm/min).
2. Exchange the buffer to 5 mL of 1× CUBIC-HV™2 Staining buffer with or without selected 1× HV-additive★ reagents and incubate it for 1.5 h~ at 37°C with gentle shaking (40-50 rpm/min).

For 5 mL of the 1× CUBIC-HV™2 Staining buffer containing a single HV-additive★ (e.g., the HV-additive★ combination 3) in **STEP 2**):

2× CUBIC-HV™2 Staining buffer	2.5 mL (final 1×)
10× HV-additives★ reagent	0.5 mL (final 1×)
Distilled water	2 mL
Total	5 mL

■ Prepare all the staining buffer recipes containing the selected combinations of HV-additive★ identified in STEP 2.

As a control, 5 mL of the 1× CUBIC-HV™2 Staining buffer without HV-additive★ is required:

2× CUBIC-HV™2 Staining buffer	2.5 mL (final 1×)
Distilled water	2.5 mL
Total	5 mL

3. During the step 2, prepare the antibody mix as follows.
   a. Calculate the required volume (X, Y) of the primary antibody and the secondary Fab fragment.
      Primary antibody: 2.5 μg/mouse brain hemisphere (approximately 0.3 g)
      To use 2.5 μg of 1 mg/mL (= 1 μg/μL) 1st Ab product, X = 2.5 (μg) /1 (μg/μL) = 2.5 μL.
      Secondary Fab fragment: 2 μg/mouse brain hemisphere
      In the case of using 1.5 mg/mL product, Y = 2 (μg)/1.5 (μg/μL) ≒ 1.33 μL.

■ When using a direct dye-conjugated primary antibody, 3-a can be skipped.

■ The quantity and final concentration of the primary antibody should be tailored to each application (usually 5 μg/whole mouse brain at a concentration of 50-100 μg/mL). However, it is advisable to begin by employing the indicated amount and concentration of antibody to initially assess HV-additive★ for 3D staining.

■ When applying to organs other than the brain, estimate the required quantity of antibodies and reagents by considering the weight ratio of the organ.

b. Prepare the following 3D staining solution (50 µL/mouse brain hemisphere).

For 50 µL of the 3D staining solution containing a single HV-additive★ reagent:

2× CUBIC-HV™2 Staining buffer	25 µL (final 1×)
Primary antibody	X µL
Secondary Fab fragment	Y µL
10× HV-additives★ reagent	5 µL
Distilled water	25 - (X+Y+5) µL
Total	50 µL

Add the entire volume to the 500 µL Protein LoBind tube and incubate the mix for 1.5 h at 37°C.

■ **The concentration of the primary antibody is 50 µg/mL (2.5 µg/50 µL) in this case.**

■ **Prepare all the staining buffer recipes containing the selected combinations of HV-additive★ identified in STEP 2.**

As a control, 50 µL of the 3D staining solution without HV-additive★ is required:

2× CUBIC-HV™2 Staining buffer	25 µL (final 1×)
Primary antibody	X µL
Secondary Fab fragment	Y µL
Distilled water	25 - (X+Y) µL
Total	50 µL

4. After the reaction in step 3, transfer the entire 3D staining solution into the 3D tissue staining pot [CSSR005].
   ■ **This 3D tissue staining pot for CUBIC-HV™2 can be used repeatedly. After the usage, wash the pot with a neutral detergent and distilled water.**
5. Collect the sample with a metal spoon and put it into the staining pot.
   ■ **A portion of the sample should be attached gently on soft paper towel to absorb residual buffer (see our general protocol for details).**
6. Slowly pour CUBIC-HV™2 Wrapping reagent into the staining pot until the pot is filled. Remove the bubbles.
   ■ **The wrapping reagent prevents samples from drying out during staining by covering the entire sample and the staining solution containing condensed staining probes (HV-LLPS★).**
7. Close the lid tightly and incubate the chamber for 3 days at RT with slow rotation (<1 rpm) under a light-shielded condition (see our general protocol for details).
8. To stabilize the secondary Fab signals, further incubate the staining chamber for 24 h at 4°C with slow rotation (<1 rpm) under a light-shielded condition.
9. 1 h before moving to the step 10, prepare 1× CUBIC-HV™2 Wash buffer and cool it on ice.

For 20 (5 × 4) mL of the 1× CUBIC-HV™2 Wash buffer:

2× CUBIC-HV™2 Wash buffer	10 mL (final 1×)
Distilled water	10 mL
Total	20 mL

10. Recover the sample from the staining pot with a metal spoon. Discard the staining solution and the wrapping reagent.
11. Remove the residual buffer and the wrapping reagent as in step 5.
12. Immerse the sample in 5 mL of pre-cooled 1× CUBIC-HV™2 Wash buffer in the 15 mL standing tube. Gently invert the tube several times to separate the wrapping reagent remaining on the sample surface.
13. Replace the 1× CUBIC-HV™2 wash buffer with a new one and continue washing the sample gently at 4°C with light shielding, while shaking at 40-50 rpm/min, for 2 h.
14. Replace the wash buffer with the new one and continue washing the sample two more times (2 h ×2).

15. During step 14, prepare ~4 mL of fixative solution by diluting formalin (FA) solution to final 1% in 1× CUBIC-HV™2 Wash buffer and cool it on ice.

■ The saturated formalin solution contains 35 to 38% of formalin. For example, when using a 37% formalin solution, dilute it with 1x CUBIC-HV™2 Wash buffer at a ratio of 1:36.

For 3.7 mL of the FA solution:

2× CUBIC-HV™2 Wash buffer	1.85 mL (final 1×)
Saturated formalin (37%)*	0.1 mL (final 1%)
*The concentration may be different by products.	
Distilled water	1.75 mL
Total	3.7 mL

16. Fix the sample in the 1% FA solution for 24 h at 4°C with gentle shaking (40-50 rpm/min) under a light-shielded condition.
17. To accelerate the fixation reaction, further incubate the sample for 1 h at 37°C with gentle shaking (40-50 rpm/min) under a light-shielded condition.

■ Reducing the FA reaction time in steps 2 and 3 could result in the attenuation of antibody signals.

18. Wash the sample in 30 mL of PBS in the 50 mL tube for 2 h at RT with gentle shaking (40-50 rpm/min) under a light-shielded condition.
19. Evaluate antibody penetration by preparing cryosections from the central regions of the sample. Begin by immercing the 3D-stained and fixed sample in 40% (w/v) sucrose/PBS solution and keep it at 4°C until the sample settles at the bottom of the tube (usually taking several hours to overnight) for cryopreservation.
20. Embed the samples in O.C.T. compound.
21. Use a cryostat to prepare sections from the central regions of the samples, cutting them at a thickness of 50 μm. Gather and wash the sections in PBS.
22. Place the sections on a slide glass for preparation and observed it with a fluorescence microscope.

■ For optimal assessment and comparison of staining signals, it is advisable to capture a complete image of the preparation, incorporating all sections, utilizing a low-magnification objective (4X) and employing the tiling function of the microscopy system.

23. Compare the staining signals in each of the tested HV-additive★ combinations with **1) No additive** condition.

−Select the best HV-additive★ combination according to the staining result.

−If required, try reducing the use of primary antibody amount with the determined HV-additive★ combination.

< Troubleshooting >

1. insufficient penetration of antibody

- extend the staining period
- increase the antibody amount and concentration

2. High background signals or aggregated signals

- reduce the antibody amount and concentration
- test other HV-additive★ combinations

3. faint or absent staining signals

- shorten the fixation period
- reduce the PFA concentration
- shorten the duration of CUBIC-L treatment
- reduce the amount of HV-additive★
- test other HV-additive★ combinations

Reference

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