CUBIC-HV

CUBIC-HV™1 3D staining protocol (version 2020.07)

[for a whole mouse brain]

CUBIC-HV™1 3D nuclear staining kit (#C3698)
CUBIC-HV™1 3D immunostaining kit (#C3699)

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MATERIALS

Reagents in kit

CUBIC-HV™1 3D nuclear staining buffer (1x) (Store at room temperature)

CUBIC-HV™1 3D immunostaining buffer (2x) (Store at 4°C; Use within 6 months)

CUBIC-HV™1 3D immunostaining wash buffer (1x) (Store at room temperature; cooled to 4°C when in use)

CUBIC-HV™1 3D immunostaining additive (10x) (included in CUBIC-HV™1 3D immunostaining kit; Store at room temperature, protected from light)

Other reagents

PBS (Tablet): TaKaRa #T9181

HEPES: Tokyo Chemical Industry #H0396

Sodium azide (NaN_3): nacalai tesque #31208-82

Paraformaldehyde: nacalai tesque #02890-45

Formalin solution: Tokyo Chemical Industry #F0622

Heparin: FUJIFILM Wako #081-00136

CUBIC-L: Tokyo Chemical Industry #T3740

CUBIC-R+: Tokyo Chemical Industry #T3741

Reagents required for the optional enzyme reaction

CAPSO: Sigma #C2278

Sodium chloride (NaCl): nacalai tesque #31319-45

Hyaluronidase: Sigma #H4272 or Sigma #H3884

BSA: Sigma #A7906

Sodium carbonate (Na_2CO_3): nacalai tesque #31310-35

Sodium hydrogen carbonate ($NaHCO_3$): nacalai tesque #31212-25

TritonX-100: nacalai tesque #12967-45

MeOH: nacalai tesque #21915-93

Nuclear staining reagents

DAPI (included in CUBIC-HV™1 3D nuclear staining kit)

BOBO™-1 Iodide: ThermoFisher Scientific B3582

SYTOX™ Green Nucleic Acid Stain: ThermoFisher Scientific S7020

Propidium Iodide (PI) (included in CUBIC-HV™1 3D nuclear staining kit)

RedDot™2 Far-Red Nuclear Stain: Biotium #40061

Antibodies

Primary antibody

According to the user's purpose*

*CUBICStars provides technical notes for a validated antibody. See our website

(https://www.cubicstars.com/cubic-hv/index.html) or email at
for request)

[NOTE] The use of antibody products with a concentration of 250 μg/mL or higher is recommended.

Secondary antibody

FabuLight Fc specific Fab fragment (Jackson Immunolab)
https://www.jacksonimmuno.com/catalog/31#target:15

[NOTE] Alexa Fluor™ 488 is not compatible with CUBIC-R+. Cy3, Alexa Fluor™ 594 and 647 have been validated.

Containers to be used

Protein LoBind 500 μL tube: Eppendorf #022431064
5 mL tube (included in CUBIC-HV™1 3D nuclear staining kit)
15 mL standing tube (included in CUBIC-HV™1 3D immunostaining kit)
30 mL tube: SARSTEDT #60.544
50 mL tube: Falcon #352070

Preparation of stock reagents

3D nuclear staining wash buffer (10 mM HEPES, pH7.5)

1M HEPES (pH 7.5)	5 mL
Distilled water	495 mL

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Total 500 mL supplied with 0.05% NaN_3

Hyaluronidase reaction buffer (pH 10)*

10 mM CAPSO (Sigma #C2278)**

150 mM NaCl

0.05% NaN_3

*The pH is critical for giving a stringent reaction condition.

**0.5 M CAPSO buffer stock:

593.3 mg of CAPSO

Distilled water

Adjust pH to 10 with NaOH

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Total 5 mL supplied with 0.05% NaN_3

[**NOTE**] The 0.5 M CAPSO buffer and the reaction buffer can be used for 2 weeks when stored at 4℃.

20 mg/mL Hyaluronidase stock (Sigma #H4272 or H3884)

Dissolve hyaluronidase in 50 mM Carbonate buffer, 150mM NaCl, 0.01% BSA* and 0.05% NaN_3** (pH 10).

*Sigma #A7906, add 1/100 volume of 1%(w/v) in water stock

**nacalai tesque #31208-82, add 1/200 volume of 10%(w/v) in water stock

[NOTE] Dispense to 77 μL (volume for a single whole brain) and store at -30°C.

Hyaluronidase wash buffer (pH 10)

50 mM Carbonate buffer*

150 mM NaCl*

0.1%(v/v) Triton X-100

5%(v/v) Methanol (nacalai tesque #21915-93)

0.05% NaN_3

*10x Carbonate buffer-NaCl stock:

2.96 g of Sodium Carbonate (nacalai tesque, #31310-35)

1.86 g of Sodium Hydrogen Carbonate (nacalai tesque, #31213-15)

8.77 g of NaCl (nacalai tesque, #31319-45)

Distilled water

Adjust pH to 10 with NaOH

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Total 100 mL, supplied with 0.05% NaN_3

Process outline

Perfusion fixation and dissection of the mouse brain

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Post-fix

↓ ~24 h

Wash (PBS)

↓ 2 h x3

Delipidation (CUBIC-L)

↓ 3~7 days

Wash (PBS)

↓ 2 h x3

Nuclear staining (HV™1 3D nuclear staining buffer)

↓ 3~5 days

Wash (3D nuclear staining wash buffer)

↓ 2 h x3 （If antibody staining is not required, skip the following steps and proceed to RI matching）

========== Optional process ==========

Exchange with the hyaluronidase reaction buffer

↓ 2 h ~

Enzyme reaction (Hyaluronidase)

↓ 24 h

Wash (Hyaluronidase wash buffer)

↓ 2 h x3

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Primary antibody + secondary antibody reaction / Replacement with HV™1 3D immunostaining buffer

↓ 1.5 h

Immunostaining [HV™1 3D immunostaining buffer with HV™1 additive (if required)]

↓ 1 week~ (up to the selected primary Ab)

Reaction at 4°C

↓ 1 day

Wash (HV™1 3D immunostaining wash buffer)

↓ 2 h x3

Post-fix

↓ 1 day

Wash (PBS)

↓ 2 h

RI matching (CUBIC-R+)

↓ ~3 days

Microscopic observation

Process details

[NOTE] Reagent volume and reaction time are indicated for staining a single whole mouse brain. It is required to adjust the volume and reaction time according to sample size.

[NOTE] All shaking steps except for enzyme reaction, immunostaining, and 4°C reaction are performed with the tube in the horizontal position.

[NOTE] Brain samples after delipidation are easily damaged and should be handled with a metal spoon.

1) Collection of mouse brain*

1. Anesthetize the mouse with an overdose of pentobarbital sodium salt (nacalai tesque #02095-04) in PBS or saline.
2. Transcardially perfuse with 10 mL (4 mL/min) of cold heparin-PBS (+10 U/mL Heparin).
3. Transcardially perfuse 20 mL (6 mL/min) of cold 4% (w/v) paraformaldehyde.
4. Dissect the brain from the skull.
5. Post-fix the dissected brain in 4% (w/v) PFA in PBS (~10 mL/whole brain) 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 x 3 times at room temperature with gentle shaking (40-50 rpm/min).

*Refer to Susaki et al. Nature Protocol 10:1709–1727 (2015) for details.

2) Delipidation

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

*If the CUBIC-L treatment is more than 3 days, replace with a new CUBIC-L every 2 to 3 days.

3. Wash the sample with 20 mL of PBS (+0.05% NaN_3) for 2 h x 3 times (or 2 h x1, overnight x1, 2 h x1) at 37°C with gentle shaking (40-50 rpm/min).

[NOTE] The tubes should be washed or replaced each time to intensively remove Triton X-100.

3) 3D nuclear staining

1. Dilute either of nuclear stains in 3-4 mL of 1x HV^{TM}1 3D nuclear staining buffer in the 5 mL tube.
   DAPI: 1/200
   BOBO-1: 1/400
   SYTOX-G: 1/2500
   PI: 1/100
   RedDot2: 1/250
2. Immerse the sample in 1x HV^{TM}1 3D nuclear staining buffer containing either of the stains. Incubate the tube with rotation (4-5 rpm) at 37°C for the following periods, protected from light.
   DAPI: 5 days

BOBO-1: 5 days
SYTOX-G: 5 days
PI: 3 days
RedDot2: 3 days

[NOTE] Increase the volume over 4 mL when the staining depth and signal intensity are insufficient.

3. Wash the sample with 15 mL of 3D nuclear staining wash buffer in the 30 mL tube protected from light for 2 h x 3 times at 25°C with gentle shaking (40-50 rpm/min).

[NOTE] If the following antibody staining is not required, proceed to 8) RI matching.

========== Optional process ==========

4) Enzyme reaction

1. Immerse the sample in 15 mL of hyaluronidase reaction buffer in the 30 mL tube protected from light for overnight at 4°C with gentle shaking (40-50 rpm/min). (or for 2 h at 37°C)
2. Prepare enzyme solution by mixing 75 μL of 20 mg/mL hyaluronidase stock into 425 μL of reaction buffer (final 3 mg/mL in 500 μL).
3. Immerse the sample in the 500 μL of enzyme solution in the 15 mL standing tube protected from light for 24 h at 37°C with gentle shaking (40-50 rpm/min).

[NOTE] To avoid damage, put the brain so that the dorsal side comes to the bottom of the tube.

[NOTE] Some antibodies are not compatible with the hyaluronidase reaction. Alternatively, Collagenase P can be used (see Nature Communications 2020 for details).

4. Wash the sample in 15 mL of hyaluronidase wash buffer in the 30 mL tube protected from light for 2 h x 3 times at 37°C with gentle shaking (40-50 rpm/min).

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5) Preparation for 3D immunostaining

Start the following preparatory tasks (1) and (2) 1.5 h prior to the staining procedure.

(1) Primary antibody + secondary antibody reaction

1. Calculate the required amount (X, Y) of primary and secondary (Fab) antibodies.

Primary antibody: 5 μg/whole brain

∗For 1 mg/mL product, X = 5/1 = 5 μL

Secondary antibody: anti-Fc Fab, 3.75 μg/whole brain (1:0.75 as the weight ratio)

∗For 1.5 mg/mL product, Y = 3.75/1.5 = 2.5 μL

[NOTE] The amount of primary antibody should be determined for each application.

[NOTE] The amount of secondary antibody should be adjusted according to the degree of noise and signal intensity.

2. Mix X μL of primary antibody and Y μL of secondary antibody in the Protein LoBind 500 μL tube. Incubate it protected from light for 1.5 h at 37°C.

(2) Exchange the immersion media with HV™1 3D immunostaining buffer.

1. Prepare 1x HV™1 3D immunostaining buffer as follows:

2x HV™1 3D immunostaining buffer	7.5 mL
Distilled water	7.5 mL
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Total	15 mL

2. Immerse the sample in 15 mL of 1x HV™1 3D immunostaining buffer in the 30 mL tube. Incubate it protected from light for 1.5 h at a specific staining temperature for the antibody with gentle shaking (40-50 rpm/min).

[NOTE] A staining temperature of 32°C is generally used. Depending on the antibody, 25°C (room temperature) or 37°C may be optimal.

6) 3D Immunostaining

1. Prepare antibody staining solution (500 μL per whole brain) as follows:

2x HV™1 3D immunostaining buffer	250 μL (final 1x)
10x HV™1 additive	50 μL (final 1x)
Distilled water	200-(X+Y) μL
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Add the entire volume to the 500 μL tube containing an X+Y μL antibody mixture.

2. Transfer the mixed antibody staining solution into the 15 mL standing tube and immerse the buffer-exchanged sample. Incubate it protected from light for 1 week~ at 32°C with gentle shaking (40-50 rpm/min).

[NOTE] Shake the tube in an upright position as during the staining.

[NOTE] To avoid damage, put the brain so that the dorsal side comes to the bottom of the tube.

[NOTE] Close the lid tightly and wrap the parafilm to prevent evaporation.

[NOTE] Staining period should be determined for each antibody.

[NOTE] The amount of HV™1 additive should be adjusted for each antibody.

3. To stabilize the 2nd Fab signals, gently shake the staining tube protected from light with a shaker (40-50 rpm/min) for a further 24 h at 4°C.

[NOTE] Shake the tube in an upright position as during the staining.

[NOTE] If the primary antibody shows a non-specific staining signal at 4°C, this step should be skipped.

4. Wash the sample in 15 mL of pre-cooled 1x HV™1 3D immunostaining wash buffer in the 30 mL tube protected from light for 30 min x 2 times at 4°C with gentle shaking (40-50 rpm/min).

[NOTE] To stabilize the binding of Fab antibodies and reduce the non-specific signal, make the reagents cooled to 4°C prior to washing and post-staining fixation operations.

[NOTE] In the case that the above 4°C reaction is skipped, wash the sample at the immunostaining temperature.

7) Post-fixation

1. Prepare the fixative solution by diluting the saturated formalin (FA) product to the final 1% in 1x HV™1 3D immunostaining wash buffer and cool it to 4°C.

   [NOTE] The saturated formalin solution contains 35 to 38% formalin. For example, when you use a 37% formalin solution, dilute it with 1x HV™1 3D immunostaining wash buffer at a ratio of 1:36.

2. Immerse the sample in 8 mL of 1% FA in the 15 mL standing tube protected from light for 24 h at 4°C with gentle shaking (40-50 rpm/min).
3. To accelerate the fixation reaction, further incubate the sample in 1% FA protected from light for 1 h at 37°C with gentle shaking (40-50 rpm/min).
4. Wash the sample in 15 mL of PBS in a 30 mL tube protected from light for 2 h at 25°C with gentle shaking (40-50 rpm/min).

8) RI matching

1. Immerse the sample in 15 mL of 0.5x CUBIC-R+ (1:1 diluted with water) in the 30 mL tube protected from light for 24 h at 25°C with gentle shaking (40-50 rpm/min).
2. Exchange with 15 mL (or 30 mL if gel embedding is performed) of 1x CUBIC-R+ for 2 days at 25°C with gentle shaking (40-50 rpm/min).
3. Use for microscopic observation (embed the sample in gel if necessary).

REFERENCES

1. Susaki et al. Versatile whole-organ/body staining and imaging based on electrolyte-gel properties of biological tissue. ***Nature Communications*** (2020) 11: 1982. DOI: 10.1038/s41467-020-15906-5
2. Matsumoto et al. Advanced CUBIC tissue clearing for whole-organ cell profiling. ***Nature Protocols*** (2019) 14: 3506–3537. DOI: 10.1038/s41596-019-0240-9
3. Susaki et al. Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging. ***Nature Protocols*** (2015) 10: 1709–1727. DOI: 10.1038/nprot.2015.085

ORDERING INFORMATION

CUBIC-HV™1 3D nuclear staining kit (Tokyo Chemical Industry #C3698)

CUBIC-HV™1 3D immunostaining kit (Tokyo Chemical Industry #C3699)

RELATED PRODUCTS

CUBIC-L (Tokyo Chemical Industry #T3740)

CUBIC-R+ (Tokyo Chemical Industry #T3741)

Formalin solution (Tokyo Chemical Industry #F0622)

Mounting Solution (RI 1.520) [for CUBIC-R+] (Tokyo Chemical Industry #M3294)

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