CUBIC-HV

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

[for a whole mouse brain]

Technical note – c-Fos staining

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

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

Anti-c-Fos: CST #2250S

[NOTE] To use this antibody, a custom product with a concentration of 200 ug/mL or higher is required.

Secondary antibody

FabuLight Fc specific Fab fragment (Jackson Immunolab)

https://www.jacksonimmuno.com/catalog/31#target:15

Fc-Fab-A594: Jackson Immuno Research #111-587-008

Fc-Fab-Cy3: Jackson Immuno Research #111-167-008

Fc-Fab-A647: Jackson Immuno Research #111-607-008

[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	

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

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

↓ 1.5 h

Immunostaining (HV™1 3D immunostaining buffer)

↓ 2-3 weeks

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.

[NOTE] Prolonged fixation or storage may result in inadequate detection of c-Fos. It is recommended to proceed to step 2) immediately after PBS wash.

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.

[NOTE] The delipidated sample can be stored in PBS/NaN_3 at 4°C.

3) 3D nuclear staining

1. Dilute either of nuclear stains in 3-4 mL of 1x HV™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™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).

4) 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: 1.8 μg/whole brain
   ∗For 0.5 mg/mL product, X = 1.8/0.25 = 7.2 μL
   Secondary antibody: anti-Mouse Fc Fab, 1.35 μg/whole brain (1:0.75 as the weight ratio)
   ∗For 1.5 mg/mL product, Y = 1.35/1.5 = 0.9 μL

[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
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 32°C with gentle shaking (40-50 rpm/min).

5) 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	5 μL (final 0.1x)
Distilled water	245-(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 3 weeks 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.

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.

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.

6) 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).

7) 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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CUBICStars

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