Version 2a, Last updated 29 April 2024

ab316248 3D Tissue Staining Kit – CUBIC

This kit enables the easy set-up of 3D tissue imaging using a simple protocol and standard laboratory equipment.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

This kit allows the practical application of a special tissue staining process that uniformly stains 3D blocks of tissue without requiring specialized equipment or reagents that are not readily accessible.

The highly reproducible protocol is compatible with various nuclear and antibody stains.

Histological staining of 3D volumetric tissue samples can be highly challenging due to complications associated with the deep penetration of staining agents and antibodies within a volumetric tissue sample. CUBIC has enabled versatile three-dimensional staining by resolving these issues. Important factors for 3D staining are the concentration of the staining probe in the buffer, and the appropriate modulation of probe-tissue interactions. Two innovative technologies address these critical factors:

CUBIC-Liquid liquid phase separation (LLPS): 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 efficient deep staining of 3D tissues.

CUBIC additives: 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 3D tissues is strongly supported.

Features

- The kit includes reagents that allow equal staining of the inside and outside of the 3D samples.
- Includes a staining pot (the staining pot can be used repeatedly and is also sold separately ab316247).
- Our simple and reproducible protocol solves issues pertaining to safety, waste processing, microscope compatibility, fluorescent protein signal retention, etc.

This product is used together with CUBIC clearing reagents (sold separately; ab316246).

To help you get started with 3D tissue staining, please visit the available data on tested antibodies on the datasheet of this product. Validated antibodies are for key markers and are tested for tissue clearing and staining with whole mouse brains. If you wish to set up tissue clearing and staining with antibodies that we haven't tested yet, please consult the protocol booklet for further details.

To learn more about tissue clearing, and tissue clearing methods (including for 3D cell cultures), please review our guide to tissue clearing here.

The tissue staining kit uses CUBIC technology.

Δ Notes:

Equally stains a whole mouse brain within a week at the shortest. Applicable for antibody and nuclear staining.

Generally, establishing 3D staining on large tissue samples poses a challenge, even for users well-versed in histological techniques. We advise initiating the application of this method with a benchmark experiment, specifically conducting anti-NeuN immunostaining with SYTOX nuclear staining on an entire mouse brain.

2. Materials Supplied and Storage

Store kit at ambient temperature on receipt. Kit can be stored for 1 year from receipt.

Item	Quantity 10 tests
10X Additive A	1 x 16 ml
10X Additive B	1 x 16 ml
10X Additive C	1 x 16 ml
10X Additive D	1 x 16 ml
10X Additive E	1 x 16 ml
10X Additive F	1 x 16 ml
1X Pre-wash buffer	1 x 150 ml
2X Staining buffer	1 x 80 ml
2X Wash buffer	1 x 300 ml
Staining Pot	1 unit
Wrapping reagent	1 x 35 ml
Casein sodium	0.8 g

Δ Notes:

Generally, the quantities of reagents required will vary depending on the size and type of organ. The reagent quantities required to stain an adult mouse brain is described in the provided example.

3. Materials Required, Not Supplied

These materials are not included in the kit but will be required to successfully perform this assay.

Materials required

Primary antibody
Secondary antibody
DAPI
Formaldehyde
Formalin
PBS
Tissue clearing reagents (CUBIC-L and CUBIC-R)

Δ Notes:

To perform tissue clearing, please purchase Tissue Clearing Kit - CUBIC (ab316246).

Before proceeding, find further details on secondary antibody requirements in the FAQ section of this booklet.

4. Reagent Preparation

The day prior to usage, dissolve casein in 2X Staining buffer through robust stirring using a stirrer.

Δ Notes:

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.

5. Sample preparation

Before staining the 3D tissue sample, the sample will have to undergo tissue clearing.

We recommend using the 3D Tissue Staining Kit - CUBIC in conjunction with Tissue Clearing Kit - CUBIC (ab316246).

We have not tested the 3D Tissue Staining Kit- CUBIC in combination with other clearing kits and can therefore not guarantee their compatibility.

6. Assay Procedure

6.1 Practical Example of Mouse Organ Staining.

Adult mouse brain after treating with CUBIC-L

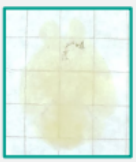
Pour the solution containing the preservative, staining probe, and staining buffer into the sample pot.

Add the sample treated with CUBIC-L and sample wrapping reagent for staining.

Set the sample in a rotator. Rotate at a speed that maintains a relative top and bottom position inside the pot without the sample flipping.

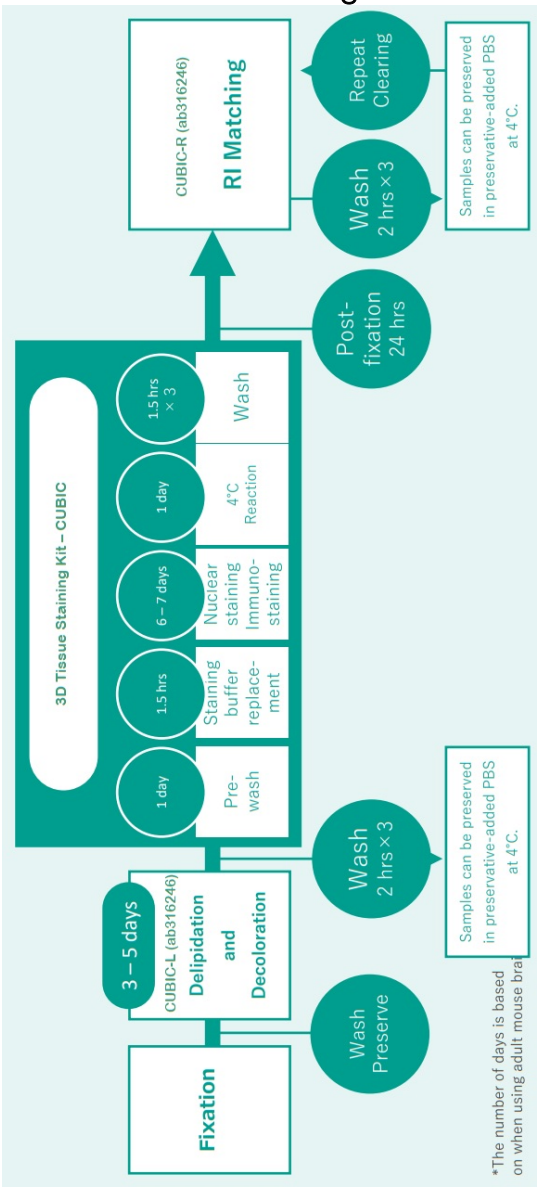
*If the sample flips inside the pot, the top and bottom of the sample will always be upward/downward in the gravity direction, resulting in unequal staining.

After staining, remove the sample from the pot and wash with the wash buffer. Shake vigorously in the first wash to remove the sample wrapping reagent.



Conduct clearing with CUBIC-R after washing and post-fixation.

6.2 Basic Process of 3D Staining Mouse Brains.



6.3 Steps to 3D Staining Mouse Brains (Basic Protocol).

Step	Reagent	Temperature	Time	Notes
Extract organ				After perfusion fixation.
Post-fixation	4% PFA in PBS	4°C	1 day	
Wash x 3	PBS	RT	Up to 2 hrs x 3	Shake gently (the same applies to the steps below).
Before replacement	50% CUBIC-L	RT Or 37°C	6 – 24 hrs	Optional step. Mix an equal amount of CUBIC-L and water.
Delipidation and Decoloration	CUBIC-L	37°C	> 2 days	Change CUBIC-L to a new solution every two days.
Wash x 3	PBS	RT	Up to 2 hrs x 3	Exchange or wash the tube every time to prevent carry-over of the solution.
Staining pre-wash	1 x 3D Tissue Staining Kit – CUBIC Pre wash buffer	37°C	Overnight	
Staining before replacement	2 x 3D Tissue Staining Kit – CUBIC Staining buffer	37°C	1.5 hrs	Add the additive. Conduct reactions of the primary antibody and secondary antibody (Fab fragment) separately during replacement.
Staining	2 x 3D Tissue Staining Kit – CUBIC Staining buffer	RT–37°C	6 – 7 days	Add the sample, additive, antibody solution, and nuclear staining reagent to the staining pot. Conduct staining in the wrapping reagent.
Reaction after staining	2 x 3D Tissue Staining Kit – CUBIC Staining buffer	4°C	Overnight	Set the reaction chamber in the previous step to 4°C as it is.
Wash x 3	2 x 3D Tissue Staining Kit – CUBIC Wash buffer	4°C	Up to 1.5 hrs x 3	Store the buffer in ice beforehand.
Post-fixation	1% Formaldehyde	4°C	1 day	Use 37% formaldehyde diluted with PBS.
Post-fixation	1% Formaldehyde	37°C	1 hr	Transfer the solution treated overnight at 4°C to the solution treated at 37°C.
Wash x 3	PBS	RT	Up to 2 hrs x 3	
Before replacement	50% CUBIC-R	RT Or 37°C	1 day	Mix an equal amount of CUBIC-L and water.
Clearing	CUBIC-R	RT Or 37°C	> 1 day	

Δ Notes:

Reagent quantities and treatment times varies depending on the organ.

Please use a slightly larger tube wider than the organ's diameter. Most of the organ should be immersed in the reagent when the tube is laid on its side.

Shake the organ immersed in the reagent at a speed that shakes the whole organ.

We do not recommend rotation as it will create bubbles.

7. Guidelines for Optimizing Antibody Concentration

If you are using an antibody for the first time, we recommend that you validate the antibody and optimize its concentration. Antibody concentration required for the workflow can be different for thicker tissues or 3D cell culture models than for thinner sections. Thicker sections require longer incubations and make workflow times longer. Therefore, we recommend that you
validate the antibody of interest using thin tissue sections first.

Δ Notes:

To check if the antibodies are compatible, compare the stains of slices before and after processing with CUBIC-L (can be purchased separately as Tissue Clearing Kit - CUBIC [ab316246).

7.1 Select primary antibodies and CUBIC-additives

Select primary antibodies (2D staining) (STEP 1)

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 staining buffer using a 24-well plate.

Δ Notes:

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 immunostaining signal.

Procedure:

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

Example: when using 1 µg/µL of primary antibody and 1.5 µg/µL of secondary Fab fragment product:

X (volume) = 2.5 µL of 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 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 500 µL Protein LoBind 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 the antibody staining solution:

1/100 dilution:

2× CUBIC 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

1/1000 dilution:

2× CUBIC Staining buffer	112.5 µL (final 1×)
Distilled water	112.5 µL
1/100-diluted staining solution (see above)	25 µL
Total	250 µL

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× Wash buffer with DAPI (1/1000) and cool it on ice.
For preparing 1× Wash buffer, dilute 1 mL of 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× 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 with 1 mL of ice-cold PBS and incubate 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 observe them 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" Evaluate suitable CUBIC additives for each primary antibody" (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 the PFA concentration or fixation time, should be considered. Try reduced PFA concentration (4% to 1 or 2%) and skip the post-fixation step.*
- *CUBIC staining 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.*

Select suitable CUBIC additives for each primary antibody (STEP 2)

CUBIC additives are probe penetration enhancers, each with distinct stringencies. Selecting an appropriate additive or combination of additives is essential for the successful CUBIC 3D staining.

Recommended 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

Δ Notes:

Combinations 2) to 5) encompass most antibody use cases, we therefore recommend starting with these combinations.

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 the antibody signal. 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).

Procedure:

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

- 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 (2D Staining evaluation).

Example: when using 1 µg/µL of primary antibody and 1.5 µg/µL of secondary Fab fragment at a 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 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 10 additive combinations).

Modify the following recipes based on the number of tested additive combinations.

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

Secondary Fab fragment	2 µL
Distilled water	18 µL
Total	20 µL (0.15 µg/µL 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 500 µL Protein LoBind 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 additives.

1) No additive (control)

2× CUBIC 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 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 Ml

3) 1x Additive A

2× CUBIC 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 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 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 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 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 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 signal is significantly reduced.

9) 1x Additive F

2× CUBIC 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 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 Wash buffer with DAPI (1/1000) and cool it on ice.

For preparing 1× CUBIC Wash buffer, dilute 1 mL of 2× CUBIC 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 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 with 1 mL of ice-cold PBS and incubate 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 for each of the tested additive combinations with the control condition.
Omit the conditions that cause higher background signals and/or significantly reduce the staining signal.

7.2 Optimize the 3D staining condition for each primary antibody with additives (STEP3)

For the final step, optimizing the 3D staining conditions, we advise to use a relatively small tissue block (e.g., a mouse brain hemisphere). Once the optimal conditions are determined, the amount of the primary antibody can be adjusted accordingly. Use of the liquid-liquid phase separation agent is integrated into this process.

Procedure:

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 as part of the tissue clearing) in 5 mL of 1× CUBIC Pre-wash buffer in a 15 mL standing tube and incubate it overnight at 37°C with gentle shaking (40-50 rpm/min).

2. Exchange the buffer with 5 mL of 1× CUBIC Staining buffer with or without selected 1× additive reagents and incubate for 1.5 h at 37°C with gentle shaking (40-50 rpm/min).

For 5 mL of the 1× CUBIC Staining buffer containing a single additive (e.g., additive combination 3):

2× CUBIC Staining buffer	2.5 mL (
10× additive reagent	0.5 mL (
Distilled water	2 mL
Total	5 mL

Prepare all the staining buffer recipes containing the selected combinations of additives identified previously (STEP 2).

As a control, 5 mL of the 1× CUBIC Staining buffer without additive is required:

2× CUBIC Staining buffer	2.5 mL (
Distilled water	2.5 mL
Total	5 mL

3. While samples incubate, prepare the antibody mix as follows.
a. Calculate the required volume of the primary antibody and the secondary Fab fragment (X, Y).

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, 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, step 3-a can be skipped.
The quantity and final concentration of the primary antibody should be tailored to each experimental setup (usually, 0.5-10 µg/whole mouse brain at a concentration of 50-100 µg/mL). We advise to initially use the indicated amount and concentration of 2.5 µg antibody/mouse brain hemisphere to assess the additives 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 additive reagent:

2× CUBIC Staining buffer	25 µL (fi
Primary antibody	X µL
Secondary Fab fragment	Y µL
10× additive reagent	
Distilled water	25-(X+Y
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 additive identified previously (STEP 2).

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

2× CUBIC Staining buffer	25 µL (fi
Primary antibody	X µL
Secondary Fab fragment	Y µL
Distilled water	25-(X+Y
Total	50 µL

4. After the reaction in step 3, transfer the entire 3D staining solution into the 3D tissue staining pot
This 3D tissue staining pot for CUBIC tissue staining can be used repeatedly. After use, 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 Wrapping reagent (phase separation reagent) into the staining pot until the pot is filled. Remove any bubbles.
The wrapping reagent prevents samples from drying out during staining by covering the entire sample and the staining solution containing condensed staining probes.

7. Close the lid tightly and incubate the chamber for 3 days at RT with slow rotation (<1 rpm) protected from light (see our general protocol for details).

8. To stabilize the secondary Fab signal, further incubate the staining chamber for 24 h at 4°C with slow rotation (<1 rpm) protected from light.

9. 1 h before moving to step 10, prepare 1× CUBIC Wash buffer and cool it on ice.

For 20 (5 × 4) mL of the 1× CUBIC Wash buffer:

2× CUBIC Wash buffer	10 mL (f
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 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 wash buffer with new buffer and continue washing the sample gently at 4°C protected from light, while shaking at 40-50 rpm/min, for 2 h.

14. Replace the wash buffer with new buffer 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 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 Wash buffer at a ratio of 1:36.

For 3.7 mL formalin solution:

2× CUBIC Wash buffer	1.85 mL
Saturated formalin (37%)*	0.1 mL (
*The concentration may be different depending on the product.	
Distilled water	1.75 mL
Total	3.7 mL

16. Fix the sample in 1% FA solution for 24 h at 4°C with gentle shaking (40-50 rpm/min) protected from light.

17. To accelerate the fixation reaction, further incubate the sample for 1 h at 37°C with gentle shaking (40-50 rpm/min) protected from light.

Reducing the FA reaction time in steps 2 and 3 could result in the reduction of antibody signal.

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) protected from light.

19. Evaluate antibody penetration by preparing cryosections from the central regions of the sample. Begin by immersing 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. Collect and wash the sections in PBS.

22. Place the sections on a slide glass for preparation and observe them with a fluorescence microscope.
For optimal assessment and comparison of staining signals, we advise 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 signal in each of the tested additive combinations with the control (no additive added).
- Select the best 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 the antibody
- extend the staining period
- increase the antibody amount and concentration

2. High background or aggregated signal

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

3. Faint or absent staining signal

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

8. Frequently Asked Questions

Questions on Staining Reagents, Antibodies, and Fluorescent Proteins.

Q: What fluorescence reagents and stains can be used with the 3D Tissue Staining Kit – CUBIC?

A: When using antibodies, we recommend using antibodies that are directly fluorescently labeled with Alexa Fluor® dyes or Fab fragment secondary antibodies. Regarding Alexa Fluor® dyes, AF488 is not compatible with CUBIC-R. Please use other dyes such as FITC instead. In the protocol, we do not recommend using general IgG secondary antibodies, as staining the depths is not guaranteed. For nuclear staining, we recommend propidium iodide (PI), SYTOXTM-Green, or RedDotTM2.

Q: Can primary antibodies available in the lab be used with the 3D Tissue Staining Kit – CUBIC?

A: In the references, some proteins that have maintained their antigenicity before and after clearing. However, not all proteins have been confirmed, so consider the antibodies at hand before use. To check if the antibodies are compatible, compare the stains of slices before and after processing with CUBIC-L.

Q: Can fluorescently labeled secondary antibodies be used after primary antibodies, similar to normal immunohistostaining?

A: For ab316248, the Fab fragment secondary antibody reacts with the primary antibody to create a complex before the 3D tissue staining. We do not recommend staining 2D slices in the general two steps (primary antibody followed by secondary antibody) because staining of the depths is not guaranteed.

Q: What fluorescent proteins can be used?

A: Fluorescent signals are maintained for EGFP, EYFP, Venus, tdTomato, mCherry, and mKate2. For other fluorescent proteins.

The protocol might require optimization for other chosen proteins.

Questions on After Clearing.

Q: The transparent sample cannot be observed well.

A: We recommend observing with light-sheet microscopes for tissue clearing observation (LSFM), confocal laser scanning microscopes (CLSM) with objective lenses compatible with high RI, and two-photon microscopes. When observing, immerse the sample in the mounting agent for observation, and use objective lenses compatible with the RIs. In recent years, affordable light-sheet microscopy for tissue clearing is developed.

Reference: descSPIM: Affordable and Easy-to-Build Light-Sheet Microscopy for Tissue Clearing Technique Users K. Otomo et al., bioRxiv. 2023

K. Otomo et al., bioRxiv. 2023
https://doi.org/10.1101/2023.05.02.539136

Q: What is the refractive index of the reagents?

A: The refractive index (RI) of CUBIC-R is 1.522. Avoid mixing solvents such as water with CUBIC-R to change the refractive index.

Δ Notes:

Regarding the information above, clearing and staining results vary depending on the transparency of the sample, staining reagents, and devices used.

Please consider the appropriate processing time and reagent concentration level.

Technical Support

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