**Step-by-step protocol of CUBIC-HistoVIsion (version 1.0) for a whole mouse brain**
(adapted from https://doi.org/10.1038/s41467-020-15906-5)
Supplementary information - https://static-content.springer.com/esm/art%3A10.1038%2Fs41467-020-15906-5/MediaObjects/41467_2020_15906_MOESM1_ESM.pdf

## Reagents

**CUBIC-L**
10 wt% N-Butyldiethanolamine (TCI #B0725)
10 wt% Triton X-100 (nacalai tesque #12967-45)
Dissolve in DDW (double distilled water)

**Staining buffer** (1x HEPES-TSC, pH 7.5)*
10 mM HEPES (nacalai tesque #17514-15)
10%(v/v) Triton X-100
200 mM NaCl
0.5%(w/v) Casein (Wako #030-01505)
0.05% NaN3
Dissolve in DDW
* For preparing antibody solution, 2x HEPES-TSC is also recommended to be prepared. Occasionally, add Quadrol [2.5~5%, prepare by diluting 50 wt% in water stock] and/or urea (0.5~2 M, nacalai tesque #35904-45) for some antibodies.

**0.5 M PB** (pH 7.5) /500 mL
30.2 g Sodium hydrogen phosphate (nacalai tesque #31726-05)
5.9 g Sodium dihydrogenphosphate dehydrate (nacalai tesque #31718-15)
0.05% NaN3
Dissolve in DDW

**0.1 M PBT**
0.1 M PB (pH 7.5)
10%(v/v) Triton X-100
0.05% NaN3
Dissolve in DDW

**1% Formaldehyde (FA)**
0.1 M PB (pH 7.5)
1% Formaldehyde (nacalai tesque #16222-65, 37% saturated formaldehyde)
Dilute 1:37 formaldehyde in PB

**CUBIC-R+** (tricky solution)
45 wt% 2,3-Dimethyl-1-phenyl-5-pyrazolone (Antipyrine, TCI #D1876)
30 wt% Nicotinamide (TCI #N0078) or N-Methylnicotinamide (TCI #M0374)
0.5%(v/v) N-Butyldiethanolamine
Dissolve in DDW

**CAUTION**: Add the water carefully. The reagents volume is very big.
*e.g. when preparing 50 ml of solution start by adding only 10 ml of water to the reagents in a 200ml glass. Dissolving takes time, you can leave it overnight*

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# Protocol

## Collection of mouse brains*

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 of cold heparin-PBS.
3. Transcardially perfuse 20 mL of cold 4% (w/v) paraformaldehyde (PFA, nacalai tesque, #02890-45).
4. Dissect the brain from the skull.
5. Post-fix the dissected brain in 4% (w/v) PFA for overnight (8-24 h) at 4°C with gentle shaking.
6. Wash the sample in PBS for 3 h x 3 times at room temperature with gentle shaking.
7. *Refer to Susaki et al. 26 for details.

## Delipidation with CUBIC-L

1. Immerse a fixed whole mouse brain in 10 mL of 0.5x CUBIC-L (1:1 dilution with water) in a 30 mL tube for overnight at room temperature with gentle shaking.* (1 mm brain slices in 3 ml).
   *This step can be skipped.
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.
3. Wash the sample in PBS for 2 h x 3 times (or 2 h x1, overnight x1, 2 h x1) at 37°C with gentle shaking. The washing tube should be replaced with a new one every time to remove Triton X-100 intensively.

## 3D nuclear staining

1. Dilute either of nuclear stains in 4 mL of ScaleCUBIC-1A with 500 mM NaCl.
   SYTOXTM Green (Thermo Fisher Scientific #S7020), 1:2500
   BOBO™-1 Iodide (462/481) (Thermo Fisher Scientific # B3582) 1:400
   RedDot™2 Far-Red Nuclear Stain (Biotium #40061), 1:150
2. Immerse the whole mouse brain in ScaleCUBIC-1A with 500 mM NaCl containing either of the stains. Keep incubation with rotation at 37°C for the following periods: SYTOX-G, BOBO-1: 5 days, RedDot2: 3 days.
3. Wash the sample with 15 mL of 10 mM HEPES (pH 7.5) in a 30 mL tube for 2 h x 3 times at 25°C with gentle shaking.

## 3D immunostaining

1. To replace the buffer, immerse the whole mouse brain in 15 mL of the staining buffer (HEPES-TSC with additives, if needed) in a 30 mL tube for 1.5 h at a specific staining temperature for each antibody. Incubate it with gentle shaking.
2. Mix a primary antibody and a secondary Fab fragment in 50 µL of HEPES-TSC (without additive) in 0.5 mL tube (1:0.5 to 1:1 as the weight ratio). Incubate it for 1.5 h at 37°C with tapping and spinning down every 30 min.
3. Prepare 500 µL* of immunostaining solution by mixing the complex of primary antibody and secondary Fab, HEPES-TSC and additives if needed.

A representative recipe is as below:

*Prepare the complex of primary antibody and secondary Fab fragment:*

| | |
|---|---|
| Primary antibody (1 mg/mL) | 5 µL (= 5 µg) |
| Secondary Fab fragment (1.5 mg/mL) | 3.3 µL (~ 5 µg for 1:1 weight ratio) |
| HEPES-TSC | 50 µL |
| | Total 58.3 µL |

Incubate it for 1.5 h at 37°C

*Prepare the immunostaining solution:*

| | |
|---|---|
| The antibody complex | 58.3 µL |
| 2x HEPES-TSC | 225 µL |
| Additive | X µL** |
| Water | the remaining volume |
| | Total 500 µL<br>(primary antibody: 5 µg in 500 µL = 10 µg/mL) |

*The volumes for smaller samples are as below:
Brain hemisphere 350 µL
Cerebral hemispheres 250 µL
Cerebellum hemisphere 120 µL

**To supply the additive, use Quadrol [by diluting 50wt% stock to final 2.5-5%(v/v)] and urea (by diluting 5 M stock to final 0.5-2 M)

4. Immerse a whole mouse brain in the immunostaining solution in a 15 mL standing tube. Keep the tube stood with gentle shaking (40-50 rpm) at a specific staining temperature for each antibody. The staining period is also dependent on each antibody (See Supplementary Table 1). To avoid the damage, put the brain so that the dorsal side comes to the bottom of the tube.
5. (optional) To improve the signal-noise ratio, incubate the sample in the immunostaining solution for 1 day~ at 4°C.
6. Wash the sample in 15 mL of 0.1 M PBT in the 30 mL tube for 30 min x 2 times at the incubated temperature with gentle shaking.
7. Wash the sample in 15 mL of 0.1 M PB in the 30 mL tube for 1 h at the same temperature with gentle shaking.

Supplementary table 1:
https://static-content.springer.com/esm/art%3A10.1038%2Fs41467-020-15906-5/MediaObjects/41467_2020_15906_MOESM4_ESM.xlsx

Postfix with 1% FA

1. Immerse the stained whole mouse brain in 1% FA in 0.1 M PB (8 mL in the 15 mL standing tube) for 24 h at 25°C with gentle shaking.
2. Wash the sample in 15 mL of 0.1M PB in a 30 mL tube for 2 h at 25°C with gentle shaking.

RI matching treatment and gel embedding

1. Immerse the whole mouse brain in 15 mL of 0.5x CUBIC-R+ (1:1 dilution with water) in a 30 mL tube for 24 h at 25°C with shaking.
2. Immerse a whole mouse brain in 30 mL of non-diluted CUBIC-R+ in 50 mL tube for 3 days at 25°C with gentle shaking.

3. After RI matching is completed, filtrate 15 mL of the used CUBIC-R+ with 5 µm filter (Millipore, #SLSVJ25LS). Store the brain sample in the remaining CUBIC-R+. Add 300 mg [2% (w/v)] of agar powder (nacalai tesque, #01163-76) into the filtrated CUBIC-R+. Completely disperse the agar powder in the reagent with a vortex.
4. Dissolve the ager by microwave. Repeat heating and mixing with vortex until the agar is completely dissolved.
5. Remove bubbles in 2% agar/CUBIC-R+ solution by incubation in a water bath for 5 min at 65°C.
6. For embedding the sample into a cleared gel, we use a Teflon mold (inside: W22 x D30 x H15 mm) of which the bottom is sealed with parafilm. Pre-heat the mold on a temperature-controlled plate (JUJI Field, LABOPAD C (COOL/HEAT), used with a custom-made black metal plate) at 45°C during step 6. Then, to make the bottom layer (approximately 2 mm thick), pour 2.5 mL of the agar solution into the pre-heated mold. Carefully remove bubbles at the surface of the gel by using a micropipette (the custom-made black metal plate and a strong light illumination help detect such small bubbles). The remaining gels should be kept in the water bath at 65°C.
7. Cool the plate for gelation for 15 min at 4°C. Then, warm the plate again for 5 min at 45°C.
8. Take out the RI-matched brain from the CUBIC-R+ and put it on a pre-heated (45°C) petri dish. Coat the brain with 1.5 mL of the agar solution.
9. Put the brain sample on the bottom layer of the gel so that the ventral side comes to the bottom. Pour the agar solution into the mold to almost cover the sample*. Remove bubbles by using a micropipette. The remaining gels should be kept in the water bath at 65°C. *An excess volume may cause the sample to float.
10. Cool the plate for gelation for 30 min at 4°C. Then, warm the plate again for 5 min at 45°C.
11. Pour a bit excess volume of the agar solution to make the top layer. Remove bubbles by using a micropipette. Then, seal the top layer of gel with a glass slide. Avoid making bubbles between the top layer of the gel and the glass slide.
12. Maintain the gel at 4°C for the complete gelation for about 1 h
13. Unmold the sample-embedding gel at room temperature and immerse it in fresh CUBIC-R+ solution. Store it in the light-shielding container.