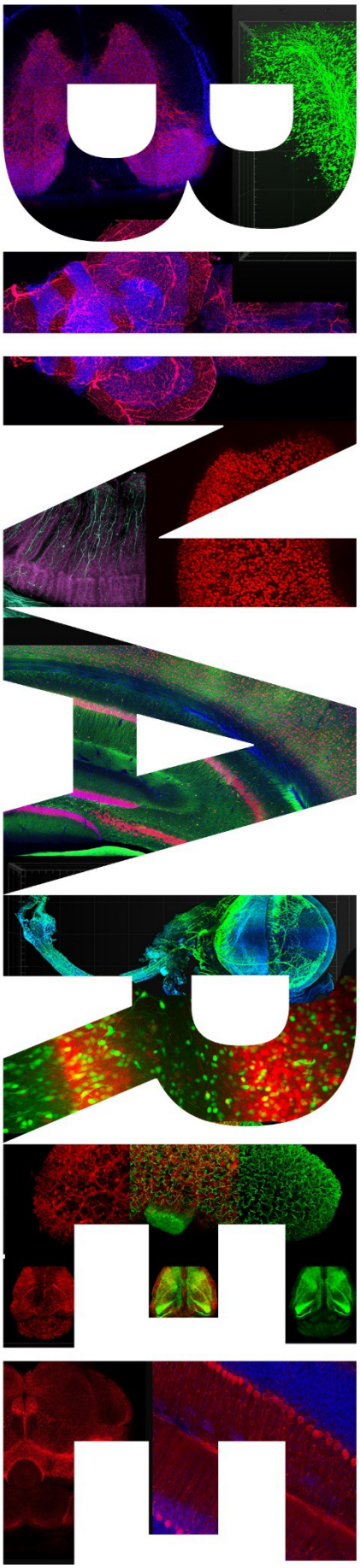

BINAREE

make visible

TISSUE CLEARING

PROTOCOL

BRTC-XXX

NAME OF PROTOCOL

Binaree® Tissue Clearing Rapid™

Cat. No. BRTC-001
Cat. No. BRTC-002

CODE OF PROTOCOL: C1003
REVISION OF PROTOCOL: 181131130

[A] - Preparation l Planning you test

Fixing → Rapid clearing → (Option) Immunostaining → Mounting → Imaging

[Tissue Clearing & Imaging within 2 days]

When we designed the protocol, we considered not only the effectiveness of the clearing but also the working time of the researchers.

Enjoy the tissue clearing!

[B] - Preparation l Taking the solutions

B-1. Tissue Clearing Rapid solution™ should be stored at -20°C and the mounting solution (BRMO-xxx) should be stored at room temperature.

B-2. Check Tissue Clearing Rapid™ Solution for crystallization or precipitation before use.

B-3. Pre-warm the Binaree Tissue Clearing Rapid ™ at 37°C 1-2 hours prior to use.

B-4. Prepare 30% (w/v) sucrose solution in 1X PBS.

B-5. Do not use the individual solutions from the other kit. Each solution has a unique component composition depending on the purpose of the kit.

B-6. BRTC-001, 002 needs to be purchased of mounting solution individually (Cat. No. BRMO-006).

[C] - Preparation l Fixing the sample

C-1. The mouse is transcardial perfused with 4% PFA.

Note. For the mouse brain perfusion, it is recommended to add **10% sucrose** in 4% PFA to increase tissue strength.

C-2. Incubate the sample with 4% PFA at 4 °C for overnight.

Note. For the mouse brain perfusion, it is recommended to add **10% sucrose** in 4% PFA to increase tissue strength.

C-3. Wash the sample with 1X PBS while shaking at 4 °C for 20 min × 3 times.

C-4. Incubate the sample with 10 ml 30 % sucrose in 1X PBS at 4 °C until the sample sinks.

NAME OF PROTOCOL | Binaree® Tissue Clearing Rapid™ | Cat. No. BRTC-001 | Cat. No. BRTC-002

[D] - Protocol l Clearing the fixed sample

Note. Incubate the Binaree Tissue Clearing Rapid™ Solution at 37 °C for 2 h before use.

D-1. In Binaree Tissue Clearing Rapid™ Chamber, transfer the sample on the bottom, press with a thick sponge to fix it and fill **20 ml Binaree Tissue Clearing Rapid™ Solution.** Place the olfactory bulb of whole brain in (+) charge of chamber. Press with a sponge to keep the mouse whole brain from moving. Also, use the enclosed cassette for thin brain slice or other tissue. For the rest of the tissue, the direction of the sample is not crucial.

D-2. Run system. Running time may vary depending on the tissue (6-8 hours/each brain).

(+) red

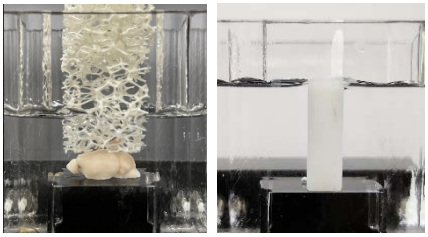

Figure 1. Place the olfactory bulb of whole brain in (+) charge of chamber. Do not press the tissue too hard with the sponge. For thin slice or other tissue, use the enclosed cassette.

D-3. (optional) Wash the sample with distilled water while shaking at 50 rpm /4 °C for 1 min.
Add nuclear stain solution (e.g. DAPI, 20-40 µg/ml in distilled water) while shaking at 4 °C for overnight.
Wash the sample with distilled water while shaking at 50 rpm /4 °C for 10 min × 3 times.

D-3. Transfer the sample to **20 ml Binaree Tissue Clearing Rapid™ Mounting Solution (BRMO-006)** and agitate at 50 rpm /24 °C (RT) for 24-48 h.

[E] - Protocol l (Option.1) Passive clearing with BRTC-XXX (without BDTC-003 device)

E-1. Proceed passive clearing after step C-4.

E-2. Transfer the sample to 20 ml Rapid tissue clearing solution (BRTC-XXX) and agitate at 50 rpm/37 °C for 7 days.

E-3. Transfer the sample to 20 ml Mounting solution (BRMO-XXX) and agitate at 50 rpm/24 °C (RT) for 2 days.

[F]- Clearing Tip

F-1. If the sample contains air bubbles → Centrifuge the sample at 3,000 rpm/24 °C for 1 min.

F-2. If the rpm is not specified → Operate the shaking incubator gently.

F-3. It is recommended to use the vial for tissue clearing rather than the chamber slide.

[G]- Storage & Imaging Tips

G-1. After imaging, the samples are stored at room temperature in the Binaree Tissue Clearing Rapid™ Mounting Solution (BRMO-006). Never refrigerate.

G-2. Take images within 7 days after the clearing for the best results.

G-3. Take images on the microscope. We recommend using a Light Sheet Fluorescence Microscope (LSFM) or Confocal Laser Scanning Microscope (CLSM).

G-4. Binaree Tissue Clearing Rapid™ Mounting Solution is safe to use in the LSFM.

G-5. Refractive Index (RI) of the Mounting Solution is 1.52.

G-6. Be careful of making bubbles while filling the microscope chamber with the sample and the Mounting Solution. The bubbles may disturb the imaging.

[H] - Protocol l (Option.2) Immunostaining for 1 mm-thick tissue

H-1. Permeabilization and Blocking

You can perform standard immunostaining procedures after clearing.

H-1-1. Incubate the sample with permeabilization solution in a shaking incubator at 37 °C for 2-3 days.

Note. You can perform standard immunostaining procedures. Permeabilization is a key step for successful antibody labeling. Therefore, the permeabilization condition should be optimized depending on the tissue and antibodies.

Note. Sample may become opaque at permeabilization step. Permeabilization solution (recommendation): 1X PBS or TBS containing 0.2-0.5% Triton X-100, 10-20% DMSO, Serum.

H-2. Antibody labeling

H-2-1. Incubate the sample with primary antibody in a shaking incubator at 37 ℃ for 3-6 days.

H-2-2. Wash the sample with 1X PBS or TBS while shaking at 4 ℃ for 20 min × 6 times.

H-2-3. Incubate the sample with secondary antibody in a shaking incubator at 37 ℃ for 3-6 days.

H-2-4. Wash the sample with 1X PBS or TBS while shaking at 4 ℃ for 20 min × 6 times.

H-2-5. (optional) Add nuclear stain solution (e.g. DAPI, 20-40 µg/ml in distilled water) while shaking at 4 ℃ for 1 h.

H-2-6. Wash the sample with 1X PBS or TBS while shaking at 4 ℃ for 10 min × 3 times.

H-2-7. Incubate the sample with 5 ml BRMO-XXX Solution in a shaking incubator at 24 ℃ (RT) for at least 12 h or more.

H-2-8. Image on a Confocal Laser Scanning Microscope.

Note. The specific depth of penetration for each antibody will be dependent upon the tissue type, the dilution, as well as the incubation time.

Note. Successful immunostaining is strongly dependent on the primary and secondary antibodies.

The followings are guidelines for successful immunostaining.

a. Antibody concentration should be optimized: Primary antibody dilution of 1 : 50 to 1 : 200 and secondary antibody dilution of 1 : 100 to 1 : 500 are required.

b. Usually, a 1 : 100 dilution of the secondary antibody works well.

c. Antibody incubation time: for 1 mm-thick tissue, 3-6 days are required.

d. Antibody dilution buffer (recommendation): 1X PBS or TBS containing 0.2-0.5% Tween 20, 5% DMSO and 5% serum.

e. In order to reduce the total immunostaining time, fluorescence conjugated primary antibody can be used.

f. In order to reduce the incubation time for the secondary antibody, you can also use an immunoglobulin fragment [e.g. Fab and $F(ab')_2$].

g. Before proceeding to a thicker tissue, immunostaining should be optimized starting with a thinner tissue.

h. To take images of tissue with less than 1 mm thickness via confocal microscope, use a slide chamber (2 wells or 4 wells) like the image below. Sealing the chamber with label tape reduces drying. Too much of the Mounting Solution can cause the sample in the chamber to shake. The optimal volume for 1 mm-thick tissue is 200 µl.

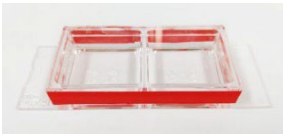

Figure 2. When taking images through confocal microscopy, the image chamber must be seal by label tape.

[I] – Appendix (For passive clearing)

Read not only the appendix but also the protocol. The protocol describes the method in detail.

I-1. Mouse ≤ 8-weeks-old

step	Summary	Temp (°C)	1 mm thickness	3 mm thickness	≤ 7 mm thickness (e.g., half brain, spleen, spinal cord)	≥ 7 mm thickness (e.g., whole brain, lung, kidney, heart)	Spheroid or Organoid
C-1	4% PFA	4°C	12 – 24 h	12 – 24 h	12 – 24 h	12 – 24 h	15 min – 1 h
C-3	Wash with 1X PBS	4°C	20 min × 3 times	20 min × 3 times	20 min × 3 times	20 min × 3 times	20 min × 3 times
C-4	30% sucrose solution*	4°C	2 – 12 h	12 – 24 h	24 h	24 – 48 h	2 – 12 h
E-2	BRTC-XXX	37°C	4 – 6 h	24 – 48 h	48 – 96 h	72 – 144 h	4 – 24 h
H-2-5	(option) DAPI staining	4°C	1 h	6 – 12 h	12 – 24 h	12 – 24 h	1 h
E-3	Mounting Solution	24°C	< 1 day	> 1 day	> 1 day	> 2 days	> 6 h

Note. *Samples were incubated in 30% (w/v) sucrose solution until the sample sanks. However, even if the sample (ex. lung, spinal cord, spheroid, etc.) does not sink in the sucrose solution after 2 days, proceed to the next steps.

[J]- Contact Us | Technical support

Binaree, Inc. (Headquarters)
○ Address: 47 Gyeongdaero17-gil Buk-gu, STE#608 IT Convergence Bldg. (115)., Daegu, 41566, Republic of Korea.
○ Website: binaree.com
○ Email: lab@binaree.com
○ Tel: +82-(0)53-939-5012Fax: +82-(0)53-382-5012